DAMPening synovial activation in osteoarthritis: taking the S100-road

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Contents

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Chapter 1

General introduction and outline of this thesis

Chapter 1: General introduction

Osteoarthritis – epidemiology

Osteoarthritis (OA) is one of the most disabling diseases in the Western world. The majority of individuals over the age of 65 have radiographic and/or clinical evidence of OA (1), which can affect hands, knees, hip and the spine. Symptoms include pain, stiffness of the joints, impaired mobility as well as signs of inflammation (2). Although OA pathology is very rarely a direct cause of death, the impact on quality of life is of particular importance. Hip and knee OA are ranked as the 11th highest contributor to global disability and the years lived with disabilities (YLDs) of OA, a parameter for the quality of life, accounts for 2.2% of total YLDs worldwide (3). The primary etiology of the disease remains unknown, however, several risk factors have been identified of which age is the most important one. Other risk factors include abnormal loading, obesity and genetic predisposition (4, 5). Due to the growing obesity epidemic as well as the increasing age, OA incidence is likely to rise considerably in the years to come (6). Current therapies for OA consist mainly of increasing mobility and controlling pain, while for end-stage OA patients health practitioners often revert to joint replacement (7).

Osteoarthritis – pathogenesis

Important pathophysiological characteristics of OA are, osteophyte formation, synovial thickening and foremost cartilage damage or even loss of cartilage (Figure 1). The progressive loss of articular cartilage leads to direct contact of bone on bone resulting in chronic joint pain and movement disabilities. Healthy articular cartilage can absorb stress and provides a smooth surface for low-friction movement of the joint. To do this, the articular cartilage consists of a unique extracellular matrix, mainly consisting of collagen type II fibers and the proteoglycan aggrecan. In this matrix, chondrocytes are embedded which balance the cartilage matrix turnover by anabolic and catabolic actions (8). One view on the cause of osteoarthritis is a failure by the chondrocytes to maintain the balance between anabolism and catabolism. A shift towards increased catabolism by chondrocytes can lead to release of pro-inflammatory factors: cytokines, damage associated molecular patterns (DAMPs)) and/or catabolic enzymes like matrix metalloproteineases (MMPs) or ADAMTSs (a disintegrin and metalloprotease

with thrombospondin motif) causing enhanced matrix degradation. Two major catabolic players thought to be involved in cartilage erosion during OA are MMP-13 and ADAMTS-5 (9, 10).

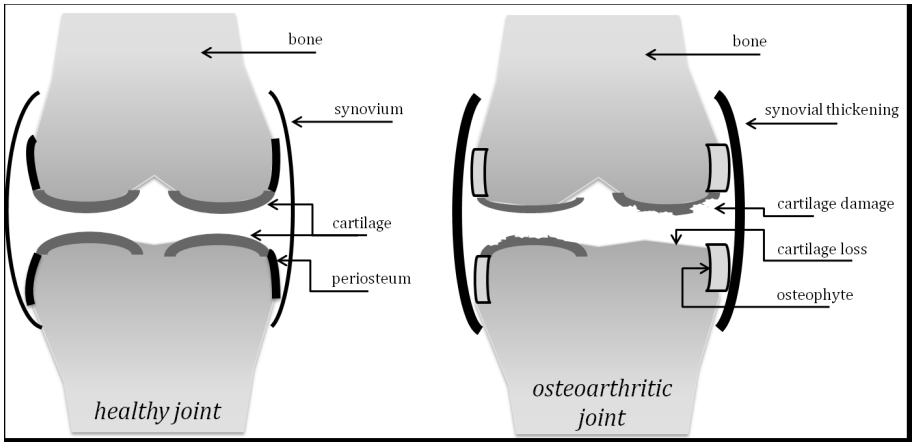

Figure 1: Pathophysiological changes in OA.

On the left a healthy joint is depicted, whereas on the right a joint with osteoarthritis, with synovial thickening, cartilage damage and loss and osteophyte formation

Other important structural changes in OA include increased subchondral plate thickness and formation of new cartilage and bone at the joint margins (osteophytes) and at tendons and ligaments (enthesophytes) (1, 11). Osteophytes or enthesophytes are cartilage-lined bony outgrowths that cause pain and limit movement in OA. Osteophytes are thought to arise from mesenchymal stem cells present in synovial lining and periosteum and are highly TGF-β and BMP-2 dependent (12, 13). Their development resembles endochondral ossification with phases of chondrogenesis, chondrocyte hypertrophy and calcification of the cartilage matrix (11, 14).

Synovial activation in osteoarthritis

Although the main focus of OA research has been on the articular cartilage and the therein residing chondrocytes, recent evidence points to the involvement of multiple tissues in the pathophysiology of OA. Pathological changes in surrounding tissue such as the synovium, bone, ligaments, subchondral bone or even musculature and meniscus have prompted clinicians and researchers to

view OA as a "whole joint" disease (15). One major focus is on the involvement of synovial inflammation or activation in OA (16). The synovium, the lining tissue of the joint capsule, consists of the intima layer containing type A macrophage-like cells and type B fibroblast-like cells and a much thicker subintima layer containing loose collagenous tissue and blood vessels. The synovium produces lubricating substances for the joint cavity and nourishes the cartilage. During OA, the intima can become thickened containing mainly monocytes/macrophages expressing an activated phenotype. Up to 50% of OA patients exhibit synovial activation as assessed by arthroscopy (17) and presence of synovitis, measured by MRI, can predict cartilage loss (18). It is thus becoming clear that OA is not one single disease and that synovial inflammation might be used as parameter to classify OA phenotypes (19). A schematic representation of three major hallmarks of OA, cartilage damage/loss, osteophyte formation and synovial activation or thickening, is depicted in Figure 1.

Macrophages in osteoarthritis

On histology, OA synovium shows hyperplasia of the lining and inflammatory infiltrate, mainly consisting of fibroblasts and macrophages (20, 21). Fibroblasts, long thought passive, have demonstrated capabilities of stimulating innate immune reactions in the synovium. Synovial fibroblasts express several Toll-like receptors (TLRs) and quickly upregulate cytokines and MMPs when stimulated with LPS (22). Macrophages have been shown to be of pivotal importance in OA. They are main producers of cytokines like IL-6 and IL-8 and enzymes MMP-1 and -3 (20). Moreover, depletion of synovial macrophages during experimental OA severely reduces cartilage damage and osteophyte formation (23, 24). Macrophages can be crudely divided into two sub-types; the pro-inflammatory M1-macrophage and the alternatively activated and less inflammatory M2-macrophage (25, 26). Presence of both subtypes has been proposed during synovitis and OA, however, their exact effect on OA pathogenesis remains enigmatic (27, 28).

Mediators of synovial activation in osteoarthritis

As a result of the synovial activation during OA, multiple mediators of innate immunity are released in the synovial fluid by macrophages, like adipokines and prostaglandins/leukotrines (29-31). Also the complement system has recently

been implicated in OA. The synovium of OA patients expresses high levels of several complement components and experimental OA studies with knockout mice show crucial roles of complement component (C)5, C6 and CD59a in OA development (32). Multiple studies have also reported elevated presence of pro-inflammatory cytokines in OA synovial tissue or fluid, among which IL-1β and TNF-α are the most studied (reviewed in (33)). These cytokines can again induce their own secretion, or drive the production of several MMPs, eventually contributing to cartilage damage (34). However, the exact contribution that these cytokines have on OA pathology is still a matter of debate.

Triggers of synovial activation in osteoarthritis: a major role for DAMPs

Apart from pro-inflammatory cytokines, the synovium can also be activated by DAMPs or alarmins. These factors can be released as a result of disruption of chondrocyte homeostasis and/or mechanical derangement. Several classes of DAMPs implicated in innate immunity are identified within the damaged OA joint, which could potentially contribute to synovial inflammation (Figure 2). Both extracellular as well as endogenous (formed within the cell) DAMPs have been described (35, 36). Examples of extracellular DAMPs are cartilage matrix breakdown products like biglycan, fibronectin, low molecular weight hyaluronic acid and tenascin C, which can stimulate cells via TLR2 (37). Joint inflammation may lead to vascular leakage and exudation of plasma proteins that can act as DAMPs as well. Recently it was found that plasma proteins α1-microglobulin and α2-macroglobulin are capable of inducing TLR4 dependent macrophage production of TNFα and IL-1β (38). Other extracellular molecules that can act as DAMPs are microscopic inorganic crystals like basic calcium phosphate (BCP) and calcium pyrophosphate dehydrate (CPPD). They are frequently observed in OA synovial fluids and tissues. At the time of joint replacement nearly all joints with severe OA show cartilage deposition of calcium-containing crystals (39). Apart from extracellular, also endogenous DAMPs are formed during OA. Examples of endogenous DAMPs are the high mobility group box 1 (HMGB1) that signals through RAGE (40), heat shock proteins (HSPs) and the family of S100-proteins (14, 41, 42). Endogenous DAMPs lead a double life, exhibiting different roles intracellularly versus extracellularly. When secreted after stimulation of cells by either stress, infection or inflammation, or released from necrotic cells, these endogenous DAMPs can activate (innate) immune cells through pattern recognition receptors (PRR) such as TLRs. They can also, depending on cell type

and their expression of PRR, stimulate cell differentiation, cell death or further secretion of pro-inflammatory mediators (36, 41).

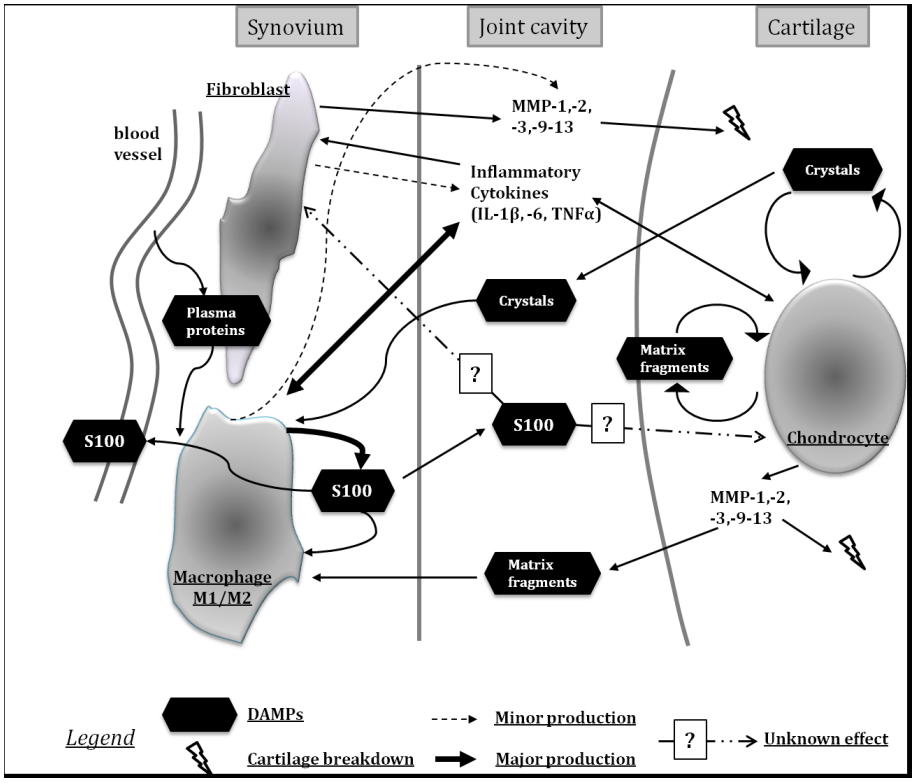

Figure 2: Effects of damage associated molecular patterns (DAMPs) in the OA joint

Overview of effects of DAMPs or alarmins on the OA joint, focusing on fibroblasts, macrophages and chondrocytes and their production of and stimulation by DAMPs.

S100A8 and S100A9

The S100-protein family currently consists of 21 known members. Its first members were originally discovered in the 1960s in brain tissue and they were named after their Solubility in 100% ammonium sulphate (43). Eleven family members so far have been identified as DAMPs or alarmins: S100B, S100P, S100A1, S100A2, S100A4, S100A7, S100A8, S100A9, S100A12, S100A13 and S100A15 (44-49). S100A8 and S100A9 (also: MRP8 and MRP14, respectively) are mainly produced by granulocytes, monocytes and activated macrophages, and can be induced in osteoclasts, endothelial cells, fibroblasts and chondrocytes (45,

50, 51). In neutrophils, S100A8 and S100A9 can form up to 40% of the cytosolic protein content (52). S100A8/S100A9 exists in vivo mostly as heterodimer (also termed calprotectin), but homodimers of both S100A8 and S100A9 exist and tetramers of two heterodimers can form under the influence of calcium and zinc cations (53). There is discussion concerning the pro-inflammatory activity of the different forms of S100A8 and S100A9, however preliminary evidence suggests that the homodimers are the most potent forms (51). Recently, it was found that the S100A8/A9 heterodimer also shows strong pro-inflammatory properties which is lost after complexing into a tetramer (Thomas Vogl, Institute of Immunology, University of Münster, personal communication). S100A8/A9 can bind calcium, a common feature of all S100-proteins (54), and intracellularly they have been shown to regulate calcium homeostasis (55). Other intracellular functions of S100A8/A9 include roles in cytoskeleton organization (56-58), fatty acid metabolism (59) and proliferation and differentiation of keratinocytes (60).

Extracellular roles of S100A8/A9

S100A8 and S100A9 can enter the extracellular space through necrosis. Although they do not possess leader signals for classical release via the Golgi-apparatus, active release has been shown for S100A8/A9 and is tubulin and protein kinase C dependent (61). Once released, S100A8 and S100A9 turn into pro-inflammatory danger signals and can stimulate a plethora of cell types. S100A8 induces the acute phase protein serum amyloid A3 (SAA3) in lung endothelial cells and alveolar macrophages (62). In human keratinocytes, S100A8 and S100A9 form a positive feedback loop with pro-inflammatory cytokines/chemokines like IL-8, -6, CXCL-1, -2 and TNFα (63). S100A8 increases TNFα and augments LPS signaling in bone marrow cells (64). More recently, S100A8 and/or S100A9 have been shown to upregulate pro-inflammatory TNFα in astrocytes, as well as IL-1β in interferon gamma (IFNγ) primed monocytes (65, 66). S100A8 and S100A9 stimulation of murine immature dendritic cells upregulates activation markers CD86 and MHC-II and induces proliferation of CD4+ and CD8+ T-cells (67). S100A8/A9 also induce chemotaxis (reviewed in (68)). In endothelial cells they increase IL-8 and CXCL-1 and -2 and decrease integrity of endothelial monolayers, implying increased attraction and extravasation of leukocytes (69). S100A8 and S100A9 also induce chemotaxis of phagocytes to a pre-metastatic lung (62).

S100A8/A9 in arthritis

It is becoming clear that DAMPs or alarmins play key roles in various (auto)-inflammatory diseases (reviewed by (41) and (70)). S100A8/A9 are found elevated in a host of inflammatory conditions, among which dermatomyositis, systemic lupus erythematosus, atherosclerosis, psoriasis and inflammatory bowel disease (71-76) and they have been postulated as biomarkers in a similarly large amount of (auto)inflammatory diseases (41, 77). Most attention however has been given to the relationship between S100A8 and S100A9 proteins and arthritis. Indeed, one of the earliest observations of S100A8 and S100A9 was in macrophages of RA patients (78). S100A8/A9 is upregulated in serum and synovial fluid of rheumatoid as well as in systemic onset juvenile idiopathic arthritis (SOJIA) and psoriatic arthritis. (36, 79, 80). Levels of S100A8/A9 in RA serum go up to 40 μg/ml and in RA synovial fluid up to a spectacular 60 μg/ml, implying local production of these DAMPs in the synovium (81). Moreover, serum levels of S100A8/A9 also correlate with disease activity in RA and psoriatic arthritis, better than the common inflammatory parameters erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (80, 82). In SOJIA, despite extensively high serum levels of IL-1β, S100A8/A9 serum levels (and not CRP) can be used for diagnosis and to distinguish SOJIA from systemic infections. In addition, S100A8/A9 can also predict development of erosive RA (83).

Mechanisms of S100A8/A9 in arthritis

Two 2008 papers by the van Lent group have shed some more light on the underlying mechanisms of action of S100A8 and S100A9 in experimental RA (51, 84). Antigen-induced arthritis was elicited in S100A9-/- mice. These mice lack functional S100A8/A9 complexes and can be viewed as a functional S100A8 and S100A9 knockout (85, 86). Arthritic S100A9 -/- mice have reduced joint swelling, as measured by technetium-99 uptake, and reduced leukocyte infiltration. Moreover, proteoglycan depletion in the cartilage was heavily reduced in S100A9 -/- mice as well as activated MMPs and chondrocyte death. The direct role of S100A8/A9 in joint damage was further established by injection of recombinant S100A8 in knee joints of normal mice. Shortly (already one day) after injection, pro-inflammatory cytokines and catabolic enzymes like MMPs and ADAMTSs were significantly upregulated, resulting in proteoglycan depletion in the cartilage. The role of the chondrocyte in S100A8/A9 mediated joint damage was

further studied in vitro. Murine chondrocytes stimulated with S100A8 (most potent in mouse) induced expression of IL-6 and increased activation of MMPs and ADAMTSs in a concentration dependent manner. The association of S100A8/A9 with cartilage and bone is further confirmed by Zreiqat et al. that showed expression of S100A8 and S100A9 in osteoclasts and hypertrophic chondrocytes (50). In a later study, the same group also confirmed inflammatory and catabolic actions of S100A8/A9 on chondrocytes by stimulating primary adult ovine articular chondrocytes. Moreover, they showed the first association of S100A8/A9 with OA, as S100A8 and S100A9 expression was observed in surgically induced mouse OA.

Osteoarthritis therapy

To date, no agent has shown to have disease modifying effects on structural progression in OA. Studies targeting synovial inflammation in OA patients are showing moderate promise. Corticosteroid injections have been shown to reduce pain in OA on the short term, however there is no evidence of long term benefits (87). Biologicals inhibiting cytokines involved in OA pathology, like TNFα and IL-1β, only have limited success. A small open label study with monoclonal antibodies against TNFα (adalumimab) showed only very modest improvement and a large placebo-controlled trial in patients with knee OA receiving IL-1 receptor antagonist (RA) (anakinra) intra-articularly showed no improvement in the WOMAC score (Western Ontario and McMaster Universities Osteoarthritis Index) (88, 89). Mesenchymal stem cells (MSCs) could also form an interesting avenue for therapy of OA. These cells combine their regenerative capacity with anti-inflammatory characteristics (90) and have been successfully used in inflammatory diseases among which autoimmune diabetes (91), experimental autoimmune encephalomyelitis (92, 93) and rheumatoid arthritis (94). MSCs can produce numerous anti-inflammatory mediators, like IL-10, IL-1RA, indoleamine 2,3-dioxygenase (IDO), transforming growth factor (TGFβ), and prostaglandin E2 (PGE2) (95). Multipotent stem cells derived from adipose tissue, adipose-derived stem cells (ASCs), have comparable differentiation capacities as MSCs and also display immunosuppressive properties in vitro and in vivo (96). An in vitro study with human OA synoviocytes and chondrocytes suggests an anti-inflammatory mechanism for ASCs (97). As such, ASCs may be used therapeutically in rheumatic diseases (98).

Outline and aims of the thesis

The general aim of this thesis was to broaden our knowledge on the role of synovial activation in OA, thereby focusing on DAMPs S100A8 and S100A9. Ultimately, we aimed to investigate ways to treat experimental osteoarthritis by tackling synovitis through inhibition of S100A8/S100A9 proteins or using anti-inflammatory ASCs.

In the first part of this thesis (**Chapter 2-6**), we investigated the role of DAMPs S100A8 and S100A9 in experimental and human OA. We started (**Chapter 2**) by mapping S100A8 and S100A9 expression in two different OA models. In the collagenase induced OA (CIOA) model synovial activation is high and involved in the pathogenesis of the disease, while the DMM model (destabilization of the medial meniscus) has considerably lower synovitis. Then, we made use of S100A9-/- mice to study the role of S100A8 (functionally absent in these mice (85, 86)) and S100A9 in synovial activation and cartilage damage in CIOA and DMM. Furthermore, in this chapter we explored the expression of S100A8/A9 in human OA synovium and their correlation with damage in the Dutch CHECK cohort (Cohort Heup En Cohort Knie) of early symptomatic OA patients.
Osteophyte formation is another important pathological hallmark of OA and the effect of DAMPs or alarmins on the development of osteophytes is poorly understood. Therefore in **Chapter 3**, we elucidated the role of DAMPs S100A8 and S100A9 in the development of osteophytes during OA. To do this, we again made use of the murine experimental OA models CIOA and DMM in the S100A9 -/- mice. To gain more insight in the mechanisms we thereafter investigated the role of S100A8/A9 in an in vitro chondrogenesis assay, as chondrogenesis is an important phase during osteophytosis. Finally, we connected S100A8/A9 levels in early symptomatic OA patients of the CHECK cohort with osteophyte development up till 5 years later.

Chondrocytes are important effector cells in OA and they balance anabolic and catabolic activity in the cartilage. Next, we investigated the up till now unknown contribution of DAMPs S100A8 and S100A9 on inflammatory and catabolic capacities of chondrocytes (**Chapter 4**). For this study we stimulated primary human OA chondrocytes with S100A8, S100A9 and S100A8/A9 complex and

analyzed expression and release of pro-inflammatory and catabolic mediators. We also sought to identify the receptor(s) responsible for these effects.
Besides chondrocytes, also macrophages play a vital part in the pathogenesis of OA, as they can initiate and sustain a pro-inflammatory and catabolic milieu. Macrophages are a heterogeneous population of cells that can be broadly divided into activatory M1 and inhibitory or anti-inflammatory M2 macrophages. In **Chapter 5**, we investigated the relationship between DAMPs S100A8 and S100A9 and M1 and M2 macrophages, by looking at the production and the stimulatory capacity of these DAMPs by the two subtypes of macrophages. Furthermore, we investigated the effect of S100A9 on total OA synovial explants, containing both macrophages and fibroblasts, and on OA fibroblasts alone.

In the final chapter of the first part (**Chapter 6**), we explored blocking DAMPs S100A8/A9 in OA for the first time. To achieve this, we used the quinoline derivative compound paquinimod (ABR-215757), that has been described to specifically inhibit the binding of S100A9 with its receptors RAGE and TLR4. We administered paquinimod in our experimental OA mouse models CIOA and DMM and analyzed OA pathology at the end stage. Moreover, we incubated synovial explants of end-stage OA patients with S100A9 and/or paquinimod and investigated the pro-inflammatory and catabolic effects.

In the second part (**Chapters 7 and 8**), we aimed to suppress synovial activation during experimental OA by local administration of adipose derived MSCs (ASCs). In **Chapter 7**, we investigated the effect of a single injection of ASCs into the knee joint during different time-points of both CIOA and DMM. We analyzed synovial activation, formation of new cartilage and bone (osteophytes) and cartilage damage in these experimental mouse models. Also, we identified the localization of the ASCs after injection in an OA model.
Next, in **Chapter 8**, we zoomed in on the mechanisms behind the effects of ASCs on experimental OA. To do this, we isolated and analyzed the synovium and its mediators of mice treated with ASCs at different time points after OA induction and focused particularly on the role of S100A8 and S100A9 in the healing capacity of these ASCs.

In the final chapter (**Chapter 9**), we summarized and discussed the results of this thesis and offered perspective on how to move forward.

References

1. Goldring SR, Goldring MB. Clinical aspects, pathology and pathophysiology of osteoarthritis. Journal of musculoskeletal & neuronal interactions. 2006;6(4):376-8.

2. Felson DT. Clinical practice. Osteoarthritis of the knee. N Engl J Med. 2006;354(8):841-8.

3. Cross M, Smith E, Hoy D, Nolte S, Ackerman I, Fransen M, et al. The global burden of hip and knee osteoarthritis: estimates from the Global Burden of Disease 2010 study. Ann Rheum Dis. 2014;73(7):1323-30.

4. Bronner F, Farach-Carson MC. Bone and osteoarthritis. London: Springer; 2007.

5. Blagojevic M, Jinks C, Jeffery A, Jordan KP. Risk factors for onset of osteoarthritis of the knee in older adults: a systematic review and meta-analysis. Osteoarthritis Cartilage. 2010;18(1):24-33.

6. Zhang Y, Jordan JM. Epidemiology of osteoarthritis. Clinics in geriatric medicine. 2010;26(3):355-69.

7. Mobasheri A. The future of osteoarthritis therapeutics: targeted pharmacological therapy. Current rheumatology reports. 2013;15(10):364.

8. Creamer P, Hochberg MC. Osteoarthritis. Lancet. 1997;350(9076):503-8.

9. Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma H-L, et al. Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature. 2005;434(7033):644-8.

10. Little CB, Barai A, Burkhardt D, Smith SM, Fosang AJ, Werb Z, et al. Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development. Arthritis Rheum. 2009;60(12):3723-33.

11. van der Kraan PM, van den Berg WB. Osteophytes: relevance and biology. Osteoarthritis Cartilage. 2007;15(3):237-44.

12. Blaney Davidson EN, Vitters EL, van Beuningen HM, van de Loo FA, van den Berg WB, van der Kraan PM. Resemblance of osteophytes in experimental osteoarthritis to transforming growth factor beta-induced osteophytes: limited role of bone morphogenetic protein in early osteoarthritic osteophyte formation. Arthritis Rheum. 2007;56(12):4065-73.

13. Bakker AC, van de Loo FA, van Beuningen HM, Sime P, van Lent PL, van der Kraan PM, et al. Overexpression of active TGF-beta-1 in the murine knee joint: evidence for synovial-layer-dependent chondro-osteophyte formation. Osteoarthritis Cartilage. 2001;9(2):128-36.

14. Goldring MB. Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. Ther Adv Musculoskelet Dis. 2012;4(4):269-85.

15. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. Bone. 2012;51(2):249-57.

16. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). Osteoarthritis Cartilage. 2013;21(1):16-21.

17. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis -- results of a 1 year longitudinal arthroscopic study in 422 patients. Osteoarthritis Cartilage. 2005;13(5):361-7.

18. Roemer FW, Guermazi A, Felson DT, Niu J, Nevitt MC, Crema MD, et al. Presence of MRI-detected joint effusion and synovitis increases the risk of cartilage loss in knees without osteoarthritis at 30-month follow-up: the MOST study. Ann Rheum Dis. 2011;70(10):1804-9.

19. Conaghan PG. Osteoarthritis in 2012: Parallel evolution of OA phenotypes and therapies. Nat Rev Rheumatol. 2013;9(2):68-70.

20. Bondeson J, Blom AB, Wainwright S, Hughes C, Caterson B, van den Berg WB. The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis. Arthritis Rheum. 2010;62(3):647-57.

21. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis. 2005;64(9):1263-7.

22. Leech MT, Morand EF. Fibroblasts and synovial immunity. Current opinion in pharmacology. 2013;13(4):565-9.

23. Blom AB, van Lent PLEM, Holthuysen AEM, van der Kraan PM, Roth J, van Rooijen N, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. Osteoarthritis Cartilage. 2004;12(8):627-35.

24. Blom AB, van Lent PL, Libregts S, Holthuysen AE, van der Kraan PM, van Rooijen N, et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. Arthritis Rheum. 2007;56(1):147-57.

25. Gordon S. Alternative activation of macrophages. Nature reviews Immunology. 2003;3(1):23-35.

26. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004;25(12):677-86.

27. Radulovic NS, Blagojevic PD, Skropeta D, Zarubica AR, Zlatkovic BK, Palic RM. Misidentification of tansy, Tanacetum macrophyllum, as yarrow, Achillea grandifolia: a health risk or benefit? Natural product communications. 2010;5(1):121-7.

28. Ambarus CA, Noordenbos T, de Hair MJ, Tak PP, Baeten DL. Intimal lining layer macrophages but not synovial sublining macrophages display an IL-10 polarized-like phenotype in chronic synovitis. Arthritis Res Ther. 2012;14(2):R74.

29. Berenbaum F, Eymard F, Houard X. Osteoarthritis, inflammation and obesity. Curr Opin Rheumatol. 2013;25(1):114-8.

30. Amin AR, Dave M, Attur M, Abramson SB. COX-2, NO, and cartilage damage and repair. Current rheumatology reports. 2000;2(6):447-53.

31. Li X, Ellman M, Muddasani P, Wang JH, Cs-Szabo G, van Wijnen AJ, et al. Prostaglandin E2 and its cognate EP receptors control human adult articular cartilage homeostasis and are linked to the pathophysiology of osteoarthritis. Arthritis Rheum. 2009;60(2):513-23.

32. Wang Q, Rozelle AL, Lepus CM, Scanzello CR, Song JJ, Larsen DM, et al. Identification of a central role for complement in osteoarthritis. Nat Med. 2011;17(12):1674-9.

33. de Lange-Brokaar BJE, Ioan-Facsinay A, van Osch GJVM, Zuurmond AM, Schoones J, Toes REM, et al. Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. Osteoarthritis Cartilage. 2012;20(12):1484-99.

34. Blom AB, van der Kraan PM, van den Berg WB. Cytokine targeting in osteoarthritis. Curr Drug Targets. 2007;8(2):283-92.

35. Busso N, So A. Microcrystals as DAMPs and their role in joint inflammation. Rheumatology (Oxford). 2012;51(7):1154-60.

36. Foell D, Wittkowski H, Roth J. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. Nat Clin Pract Rheumatol. 2007;3(7):382-90.

37. Erridge C. Endogenous ligands of TLR2 and TLR4: agonists or assistants? J Leukoc Biol. 2010;87(6):989-99.

38. Sohn DH, Sokolove J, Sharpe O, Erhart JC, Chandra PE, Lahey LJ, et al. Plasma proteins

present in osteoarthritic synovial fluid can stimulate cytokine production via Toll-like receptor 4. Arthritis Res Ther. 2012;14(1):R7.

39. MacMullan P, McMahon G, McCarthy G. Detection of basic calcium phosphate crystals in osteoarthritis. Joint, bone, spine : revue du rhumatisme. 2011;78(4):358-63.

40. Kierdorf K, Fritz G. RAGE regulation and signaling in inflammation and beyond. J Leukoc Biol. 2013;94(1):55-68.

41. Chan JK, Roth J, Oppenheim JJ, Tracey KJ, Vogl T, Feldmann M, et al. Alarmins: awaiting a clinical response. J Clin Invest. 2012;122(8):2711-9.

42. Mobasheri A. The future of osteoarthritis therapeutics: emerging biological therapy. Current rheumatology reports. 2013;15(12):385.

43. Moore BW. A soluble protein characteristic of the nervous system. Biochem Biophys Res Commun. 1965;19(6):739-44.

44. Fritz G. RAGE: a single receptor fits multiple ligands. Trends in biochemical sciences. 2011;36(12):625-32.

45. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol. 2009;86(3):557-66.

46. Towheed TE, Hochberg MC. A systematic review of randomized controlled trials of pharmacological therapy in osteoarthritis of the knee, with an emphasis on trial methodology. Semin Arthritis Rheum. 1997;26(5):755-70.

47. Rohde D, Schon C, Boerries M, Didrihsone I, Ritterhoff J, Kubatzky KF, et al. S100A1 is released from ischemic cardiomyocytes and signals myocardial damage via Toll-like receptor 4. EMBO molecular medicine. 2014;6(6):778-94.

48. Leclerc E, Fritz G, Vetter SW, Heizmann CW. Binding of S100 proteins to RAGE: an update. Biochim Biophys Acta. 2009;1793(6):993-1007.

49. Van Crombruggen K, Jacob F, Zhang N, Bachert C. Damage-associated molecular patterns and their receptors in upper airway pathologies. Cellular and molecular life sciences : CMLS. 2013;70(22):4307-21.

50. Zreiqat H, Howlett CR, Gronthos S, Hume D, Geczy CL. S100A8/S100A9 and their association with cartilage and bone. J Mol Histol. 2007;38(5):381-91.

51. van Lent PLEM, Grevers LC, Blom AB, Arntz OJ, van de Loo FAJ, van der Kraan P, et al. Stimulation of chondrocyte-mediated cartilage destruction by S100A8 in experimental murine arthritis. Arthritis Rheum. 2008;58(12):3776-87.

52. Edgeworth J, Freemont P, Hogg N. Ionomycin-regulated phosphorylation of the myeloid calcium-binding protein p14. Nature. 1989;342(6246):189-92.

53. Vogl T, Leukert N, Barczyk K, Strupat K, Roth J. Biophysical characterization of S100A8 and S100A9 in the absence and presence of bivalent cations. Biochim Biophys Acta. 2006;1763(11):1298-306.

54. Yammani RR. S100 proteins in cartilage: role in arthritis. Biochim Biophys Acta. 2012;1822(4):600-6.

55. McNeill E, Conway SJ, Roderick HL, Bootman MD, Hogg N. Defective chemoattractant-induced calcium signalling in S100A9 null neutrophils. Cell Calcium. 2007;41(2):107-21.

56. Vogl T, Ludwig S, Goebeler M, Strey A, Thorey IS, Reichelt R, et al. MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. Blood. 2004;104(13):4260-8.

57. Roth J, Burwinkel F, van den Bos C, Goebeler M, Vollmer E, Sorg C. MRP8 and MRP14, S-100-like proteins associated with myeloid differentiation, are translocated to plasma membrane and intermediate filaments in a calcium-dependent manner. Blood.

1993;82(6):1875-83.

58. Goebeler M, Roth J, van den Bos C, Ader G, Sorg C. Increase of calcium levels in epithelial cells induces translocation of calcium-binding proteins migration inhibitory factor-related protein 8 (MRP8) and MRP14 to keratin intermediate filaments. Biochem J. 1995;309 (Pt 2):419-24.

59. Kerkhoff C, Vogl T, Nacken W, Sopalla C, Sorg C. Zinc binding reverses the calcium-induced arachidonic acid-binding capacity of the S100A8/A9 protein complex. FEBS Lett. 1999;460(1):134-8.

60. Voss A, Bode G, Sopalla C, Benedyk M, Varga G, Bohm M, et al. Expression of S100A8/A9 in HaCaT keratinocytes alters the rate of cell proliferation and differentiation. FEBS Lett. 2011;585(2):440-6.

61. Rammes A, Roth J, Goebeler M, Klempt M, Hartmann M, Sorg C. Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. J Biol Chem. 1997;272(14):9496-502.

62. Hiratsuka S, Watanabe A, Sakurai Y, Akashi-Takamura S, Ishibashi S, Miyake K, et al. The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. Nature cell biology. 2008;10(11):1349-55.

63. Nukui T, Ehama R, Sakaguchi M, Sonegawa H, Katagiri C, Hibino T, et al. S100A8/A9, a key mediator for positive feedback growth stimulation of normal human keratinocytes. J Cell Biochem. 2008;104(2):453-64.

64. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007;13(9):1042-9.

65. Koy M, Hambruch N, Hussen J, Pfarrer C, Seyfert HM, Schuberth HJ. Recombinant bovine S100A8 and A9 enhance IL-1beta secretion of interferon-gamma primed monocytes. Veterinary immunology and immunopathology. 2013;155(3):162-70.

66. Omran A, Ashhab MU, Gan N, Kong H, Peng J, Yin F. Effects of MRP8, LPS, and lenalidomide on the expressions of TNF-alpha , brain-enriched, and inflammation-related microRNAs in the primary astrocyte culture. TheScientificWorldJournal. 2013;2013:208309.

67. Petersen B, Wolf M, Austermann J, van Lent P, Foell D, Ahlmann M, et al. The alarmin Mrp8/14 as regulator of the adaptive immune response during allergic contact dermatitis. Embo J. 2013;32(1):100-11.

68. Ryckman C, McColl SR, Vandal K, de Medicis R, Lussier A, Poubelle PE, et al. Role of S100A8 and S100A9 in neutrophil recruitment in response to monosodium urate monohydrate crystals in the air-pouch model of acute gouty arthritis. Arthritis Rheum. 2003;48(8):2310-20.

69. Viemann D, Strey A, Janning A, Jurk K, Klimmek K, Vogl T, et al. Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. Blood. 2005;105(7):2955-62.

70. Manfredi AA, Capobianco A, Bianchi ME, Rovere-Querini P. Regulation of dendritic- and T-cell fate by injury-associated endogenous signals. Critical reviews in immunology. 2009;29(1):69-86.

71. Foell D, Roth J. Proinflammatory S100 proteins in arthritis and autoimmune disease. Arthritis Rheum. 2004;50(12):3762-71.

72. Haga HJ, Brun JG, Berntzen HB, Cervera R, Khamashta M, Hughes GR. Calprotectin in patients with systemic lupus erythematosus: relation to clinical and laboratory parameters of disease activity. Lupus. 1993;2(1):47-50.

73. Foell D, Wittkowski H, Ren Z, Turton J, Pang G, Daebritz J, et al. Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during

inflammatory bowel disease. The Journal of pathology. 2008;216(2):183-92.

74. Averill MM, Kerkhoff C, Bornfeldt KE. S100A8 and S100A9 in cardiovascular biology and disease. Arteriosclerosis, thrombosis, and vascular biology. 2012;32(2):223-9.

75. Kerkhoff C, Voss A, Scholzen TE, Averill MM, Zanker KS, Bornfeldt KE. Novel insights into the role of S100A8/A9 in skin biology. Experimental dermatology. 2012;21(11):822-6.

76. Seeliger S, Vogl T, Engels IH, Schroder JM, Sorg C, Sunderkotter C, et al. Expression of calcium-binding proteins MRP8 and MRP14 in inflammatory muscle diseases. Am J Pathol. 2003;163(3):947-56.

77. van Bon L, Cossu M, Loof A, Gohar F, Wittkowski H, Vonk M, et al. Proteomic analysis of plasma identifies the Toll-like receptor agonists S100A8/A9 as a novel possible marker for systemic sclerosis phenotype. Ann Rheum Dis. 2014;73(8):1585-9.

78. Odink K, Cerletti N, Bruggen J, Clerc RG, Tarcsay L, Zwadlo G, et al. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. Nature. 1987;330(6143):80-2.

79. Frosch M, Roth J. New insights in systemic juvenile idiopathic arthritis--from pathophysiology to treatment. Rheumatology (Oxford). 2008;47(2):121-5.

80. Kane D, Roth J, Frosch M, Vogl T, Bresnihan B, FitzGerald O. Increased perivascular synovial membrane expression of myeloid-related proteins in psoriatic arthritis. Arthritis Rheum. 2003;48(6):1676-85.

81. Sunahori K, Yamamura M, Yamana J, Takasugi K, Kawashima M, Yamamoto H, et al. The S100A8/A9 heterodimer amplifies proinflammatory cytokine production by macrophages via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase in rheumatoid arthritis. Arthritis Res Ther. 2006;8(3):R69.

82. Hammer HB, Odegard S, Fagerhol MK, Landewe R, van der Heijde D, Uhlig T, et al. Calprotectin (a major leucocyte protein) is strongly and independently correlated with joint inflammation and damage in rheumatoid arthritis. Ann Rheum Dis. 2007;66(8):1093-7.

83. Liao H, Wu J, Kuhn E, Chin W, Chang B, Jones MD, et al. Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. Arthritis Rheum. 2004;50(12):3792-803.

84. van Lent PLEM, Grevers L, Blom AB, Sloetjes A, Mort JS, Vogl T, et al. Myeloid-related proteins S100A8/S100A9 regulate joint inflammation and cartilage destruction during antigen-induced arthritis. Ann Rheum Dis. 2008;67(12):1750-8.

85. Hobbs JA, May R, Tanousis K, McNeill E, Mathies M, Gebhardt C, et al. Myeloid cell function in MRP-14 (S100A9) null mice. Mol Cell Biol. 2003;23(7):2564-76.

86. Manitz M-P, Horst B, Seeliger S, Strey A, Skryabin BV, Gunzer M, et al. Loss of S100A9 (MRP14) results in reduced interleukin-8-induced CD11b surface expression, a polarized microfilament system, and diminished responsiveness to chemoattractants in vitro. Mol Cell Biol. 2003;23(3):1034-43.

87. Ringdahl E, Pandit S. Treatment of knee osteoarthritis. American family physician. 2011;83(11):1287-92.

88. Verbruggen G, Wittoek R, Vander Cruyssen B, Elewaut D. Tumour necrosis factor blockade for the treatment of erosive osteoarthritis of the interphalangeal finger joints: a double blind, randomised trial on structure modification. Ann Rheum Dis. 2012;71(6):891-8.

89. Chevalier X, Goupille P, Beaulieu AD, Burch FX, Bensen WG, Conrozier T, et al. Intraarticular injection of anakinra in osteoarthritis of the knee: a multicenter, randomized, double-blind, placebo-controlled study. Arthritis Rheum. 2009;61(3):344-52.

90. Maumus M, Guerit D, Toupet K, Jorgensen C, Noel D. Mesenchymal stem cell-based therapies in regenerative medicine: applications in rheumatology. Stem cell research & therapy. 2011;2(2):14.

91. Bassi EJ, Moraes-Vieira PM, Moreira-Sa CS, Almeida DC, Vieira LM, Cunha CS, et al. Immune regulatory properties of allogeneic adipose-derived mesenchymal stem cells in the treatment of experimental autoimmune diabetes. Diabetes. 2012;61(10):2534-45.

92. Rafei M, Campeau PM, Aguilar-Mahecha A, Buchanan M, Williams P, Birman E, et al. Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner. J Immunol. 2009;182(10):5994-6002.

93. Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood. 2005;106(5):1755-61.

94. Gonzalez-Rey E, Gonzalez MA, Varela N, O'Valle F, Hernandez-Cortes P, Rico L, et al. Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis. Ann Rheum Dis. 2010;69(1):241-8.

95. Pittenger M. Sleuthing the source of regeneration by MSCs. Cell stem cell. 2009;5(1):8-10.

96. De Toni F, Poglio S, Youcef AB, Cousin B, Pflumio F, Bourin P, et al. Human adipose-derived stromal cells efficiently support hematopoiesis in vitro and in vivo: a key step for therapeutic studies. Stem cells and development. 2011;20(12):2127-38.

97. Manferdini C, Maumus M, Gabusi E, Piacentini A, Filardo G, Peyrafitte JA, et al. Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2. Arthritis Rheum. 2013;65(5):1271-81.

98. Djouad F, Bouffi C, Ghannam S, Noel D, Jorgensen C. Mesenchymal stem cells: innovative therapeutic tools for rheumatic diseases. Nat Rev Rheumatol. 2009;5(7):392-9.

Chapter 2

Active involvement of alarmins S100A8 and S100A9 in the regulation of synovial activation and joint destruction during mouse and human osteoarthritis

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Chapter 2

Active involvement of alarmins S100A8 and S100A9 in the regulation of synovial activation and joint destruction during mouse and human osteoarthritis

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Abstract

Objective: To investigate whether alarmins S100A8 and A9 are involved in mediating cartilage destruction during murine and human osteoarthritis (OA).

Methods: Two different murine models of OA that differed in terms of synovial activation were compared. Cartilage destruction was measured histologically. Synovial biopsy and serum samples from OA patients were derived from the Cohort Hip and Cohort Knee (CHECK) patients with symptomatic early OA. Expression of mediators in the synovium was measured by reverse transcription–polymerase chain reaction analysis and immunolocalization

Results: In collagenase-induced OA, which showed marked synovial activation, interleukin-1 was expressed at significant levels only during the early stages of disease, whereas S100A8 and S100A9 expression remained high for a prolonged period of time (up to day 21 after induction). In S100A9-knockout mice, we found a major impact of S100A8 and S100A9 on synovial activation (62% inhibition) and OA cartilage destruction (45–73% inhibition) as compared to wild-type controls. In contrast, in the surgically induced destabilized medial meniscus model, in which synovial involvement is scant, we found no role of S100A8 and S100A9 in the focal OA cartilage destruction. Examination of arthroscopic synovial biopsy samples from patients in the early symptomatic OA CHECK cohort revealed substantial levels of S100A8 and S100A9 messenger RNA and protein, which correlated significantly with synovial lining thickness, cellularity in the subintima, and joint destruction. Levels of S100A8/A9 serum protein were significantly enhanced (19%) at baseline in patients who had pronounced progression of joint destruction after 2 years.

Conclusions: Our data suggest that the S100A8 and S100A9 proteins are crucially involved in synovial activation and cartilage destruction during OA and that high levels may predict joint destruction in humans with OA.

Introduction

Breakdown of the cartilage matrix is one of the hallmarks of osteoarthritis (OA). Cartilage destruction is predominantly mediated by cytokines and enzymes (1-4). During osteoarthritis a clear pericellular activation of chondrocytes is observed expressing large amounts of neoepitopes induced by metalloproteinases which drive pericellular breakdown of the matrix eventually culminating in erosion (3). Pathology that develops during OA is driven by at least three different tissues: cartilage, subchondral bone and synovium (5-6). Although OA has been generally described as a disease of the cartilage, the synovium is considered to contribute to pathology during progression of the disease (7-11). A substantial group of OA patients (up to 50%) show activation of the synovium, not only during late phases but also in early stages of the disease (8). In earlier studies we showed that activated synovial lining macrophages are crucial in regulating synovial inflammation and subsequent cartilage destruction during experimental OA. Selective elimination of synovial lining macrophages prior to induction of collagenase-induced OA diminished synovial activation and almost completely inhibited cartilage destruction (12).

Activated macrophages which cover the inside of diarthrodial joints produce a plethora of mediators, among them cytokines like interleukin-1β (IL-1β) (2). Although IL-1β has been considered important in mediating cartilage destruction (2,13), its role in OA is still a matter of debate. The most prominent proteins released by activated macrophages are the calgranulins: myeloid related proteins MRP8 [S100A8] and MRP14 [S100A9] (14-15). These proteins belong to the group of Damage Associated Molecular Pattern proteins (DAMPs) which are crucial in innate immunity. Both proteins belong to the S100 family of calcium binding proteins which comprises 24 members. They are expressed as homodimers and heterodimer assemblies. S100A8 is generally co-expressed with S100A9 and the heterodimer S100A8/A9 is translocated to membrane and cytoskeletal structures upon activation (16). In mice, S100A8 forms the active part whereas S100A9 forms the regulating unit which binds to S100A8 thereby preventing its degradation. S100A8 stimulates macrophages via TLR4 signalling (17-18). When secreted, S100A8 exhibits pro-inflammatory functions leading to activation of endothelial cells and phagocytes.

Homodimers S100A8 and A9 and the heterodimer accumulate in inflammatory fluids during arthritis (18-19) and their levels correlate significantly with severity of arthritis and predicted a 10 year radiographic progression in RA patients (20). In earlier studies we found that S100A8/S100A9 is crucial in mediating cartilage destruction during experimental arthritis (21). S100A8 and A9 have recently also been described in experimental osteoarthritis (22) and S100A8 appeared to be a potent stimulator of murine chondrocytes thereby inducing a catabolic phenotype (23). The goal of the present study was to investigate whether these proteins are involved in synovial activation and cartilage destruction in experimental osteoarthritis, using two murine models which differ in degree of synovial activation. In addition, we explored the clinical relevance of S100A8/A9 in synovial biopsies and serum from participants of the early OA symptomatic CHECK cohort.

Material and methods

Animals

Experimental osteoarthritis was elicited in male C57Bl/6 mice and S100A9-/- mice backcrossed to the C57BL/6 background for 12 generations. Myeloid cells of S100A9-/- also lack S100A8 at the protein level (24).

Induction of experimental osteoarthritis

Collagenase osteoarthritis (CIO) was induced by injecting 5 mg of bacterial Collagenase (Sigma Aldrich, Zwijndrecht, The Netherlands) two times at alternate days into knee joints of mice (C57BL/6) which causes disruption of the ligaments and local instability of the knee joint (25). DMM osteoarthritis was induced by transsection of the medial anterior meniscotibial ligament which leads to destabilization of the medial meniscus (DMM) (26). Experimental arthritis was induced by injection of 25 μg of streptococcal cell walls (SCW) into the murine knee joint (27).

Histology of total knee joints

Total knee joint sections of mice were fixed, dehydrated and embedded in paraffin. Sections (7 μM) were cut and stained with haematoxylin/eosin (H&E) or safranin-O. H&E-staining was used to score synovial activation as thickening of the intima lining layer (number of cell layers). Safranin-O staining was used to examine and score the OA-like cartilage changes, in which both severity and extent of the lesions were incorporated. The scoring method as described by Pritzker et al (28) was modified to make it more suitable for measuring murine cartilage pathology. In short, the OA score is the assessment of combined OA grade and stage. Grade represents OA depth progression into cartilage and includes six grades: 1-4 involve cartilage changes only;5 and 6 involve bone as well. The stage represents the horizontal extent of cartilage involvement irrespective of the underlying grade: thereby four stage stages are distinguished: 1:<10%, 2:0-<25%, 3:25-50%,4: >50%.

Immunohistochemical detection of S100A8/A9 protein

Whole knee joint sections were incubated for two hours with 10 mM citrate (pH 6.0) and thereafter incubated for 1 hour with the primary antibody directed against S100A8 or S100A9 (made in our own facilities). Rabbit IgG antibody was used as a control. After rinsing, sections were incubated for 30 minutes with biotinylated horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark). Development of the peroxidase staining was done with diaminobenzidine. Counterstaining was done with Mayer's haematoxylin (Biochemica, Amsterdam, The Netherlands).

Isolation of murine synovial specimen

At various time-points after induction of osteoarthritis, synovial specimen were isolated as described previously (29). In short, joint capsule specimens were isolated on medial and lateral sides of the patella with a biopsy punch. Synovial specimen were snap frozen in liquid nitrogen and stored for RNA isolation.

Preparation of RNA and RT-PCR

RNA was isolated from synovium using TRIzol reagent according to the

manufacturer's protocol (Invitrogen Corp.). Five micrograms of total RNA was reverse transcribed, and cDNA aliquots were subjected to PCR. RT-PCR was normalized by the transcriptional levels of GAPDH. mRNA levels of various members of the S100 family (S100B, A2, A4, A6, A7, A8, A9 and A11) and cytokines/ chemokines (IL-1β, IL-6, TNFα, IL-10, KC and MIP-1α) were measured in synovial specimen at various time-points after induction of collagenase-induced or DMM osteoarthritis and were quantified using the ABI/PRISM 7000 Sequence Detection System.

Patients and tissue preparation

Synovial tissue was obtained from patients with OA (n=10) who were undergoing arthroplasty. Furthermore biopsies were taken arthroscopically from participants with knee pain (n=19) that entered the CHECK cohort. Biopsies were taken from various areas of the synovium to minimize bias taken from inflamed tissue rather than globally. Patients undergoing arthroplasty met the American College of Rheumatology classification criteria for OA (30). CHECK (Cohort Hip and Cohort Knee) is a prospective cohort study of 1002 individuals with early symptomatic OA of knee or hip (31). On entry, all participants had pain or stiffness of knee or hip, and were aged 45-65 years. They had not yet consulted their physician for these symptoms, or the first consultation was within 6 months before entry. Participants with any other pathological condition that could explain the symptoms were excluded (e.g.other rheumatic disease, previous hip or knee joint replacement, congenital dysplasia, osteochondritis dissecans, intra-articular fractures, septic arthritis, Perthes' Disease, ligament or meniscus damage, plica syndrome, Baker's cyst). Joint destruction was determined at baseline and 2 years after the start of the study, using the Kellgren & Lawrence score of radiographs. The sum of the Kellgren & Lawrence score for both hips and knee joints was calculated. Two patient groups were defined: [1] Non-progressors. Patients that showed neither joint abnormalities (Kellgren & Lawrence=0) at baseline nor after 2 years and [2] Progressors. Patients that did not show joint abnormalities at baseline, but did show joint damage using the Kellgren & Lawrence score after 2 years (K&L ≥ 1).

Immunolocalisation of S100A8 and S100A9 protein in synovium of early symptomatic OA patients

Tissues were fixed in 10% buffered formalin. Five micron sections were stained with rabbit-anti-human S100A8 and S100A9 (made in our own facilities) or isotype control and then incubated with biotinylated rabbit anti-goat antibody (Dako, Glostrup, Denmark). Sections were treated with streptavidine –peroxidase conjugate (Millipore, Amsterdam, The Netherlands), color was visualized with hematoxylin.

Measurement of S100A8/A9 in sera of early symptomatic OA patients

Serum was kept at –20°C and S100A8/A9 serum concentrations were determined by a sandwich enzyme linked immunosorbent assay (ELISA) system as described previously (32). For calibration different amounts (0.25–250 ng/ml) of the native complex of human S100A8/A9 were used, which were isolated from human granulocytes. The assay has a sensitivity of <0.5ng/ml and a linear range between 1 and 30 ng/ml. S100A8 and S100A9 form non-covalently associated complexes, which are detected by the sandwich ELISA system. Therefore the ELISA is calibrated with the native S100A8/A9 complex and the data are expressed as ng/ml S100A8/A9. The serum samples were coded, and the results represent the mean of duplicates of each of three dilutions within the linear range.

Statistical analysis

Data were statistically evaluated using the Mann Whitney U test and spearman correlation. Confounding by BMI was excluded by multiple regression analysis.

Ethical approvement of experiments

All animal experiments were approved by the local ethics commission of the Radboud University Nijmegen Medical Centre (RU-DEC 2008-162). The human study was approved by the medical ethics committees of all participating centers, and all participants gave their written informed consent before entering the study.

Results

Clear synovial activation in collagenase-induced, but not destabilized medial meniscus–induced, OA

In a considerable number of OA patients synovial activation is observed, characterized by a thickened intima containing hypertrophic macrophages (33). We evaluated the course and degree of synovial activation at various time-points after induction of 2 models of instability related murine OA. In control murine knee joints the synovial intima is only one layer thick, comprising type A macrophage-like and type B fibroblast-like cells (Figure 1B,C). In the collagenase-induced OA, a clear thickening of the intima was seen (Figure 1D,E), mainly consisting of hypertrophic F4/80 vesicular type A macrophage-like cells (Figure 1H versus control Figure 1I). Thickening of the intima lining layer containing 3-12 layers was observed throughout the 42 days observation period, reaching its maximum at day 14 (Figure 1A). In contrast, in the DMM model, synovial activation was much lower than in the collagenase-induced OA and reached its maximum at day 7 only expressing an intima layer of maximal 2-3 layers consisting of type A macrophages which showed a less pronounced hypertrophic character (Figure 1F,G).

High and prolonged expression of S100A8 and S100A9 in the synovium in experimental collagenaseinduced OA

We next investigated whether synovia produce S100 proteins. RNA was isolated from synovia derived from collagenase-induced knee joints at several time-points (7, 21 and 42 days after induction). mRNA levels of most S100 proteins were strongly upregulated in synovium of collagenase-induced osteoarthritis when compared to control synovium, with S100A8 and A9 being the most upregulated. At day 7 and 21 expression of S100A8 was increased 24 and 32 fold and for S100A9 16 and 64 fold (Figure 2A). Interestingly mRNA levels of S100A9 increased during the course of OA up till day 21, whereas S100A8 levels remained at a high level from day 7 to day 21. In contrast, all other S100 members had returned to low levels at day 21.

In addition, mRNA levels of S100 members were measured in the synovium during the course of DMM in which less synovial activation was observed. In contrast to the levels found in CIO, DMM showed only minor increases except for S100A8 and S100A9 at day 7 (Figure 2B).

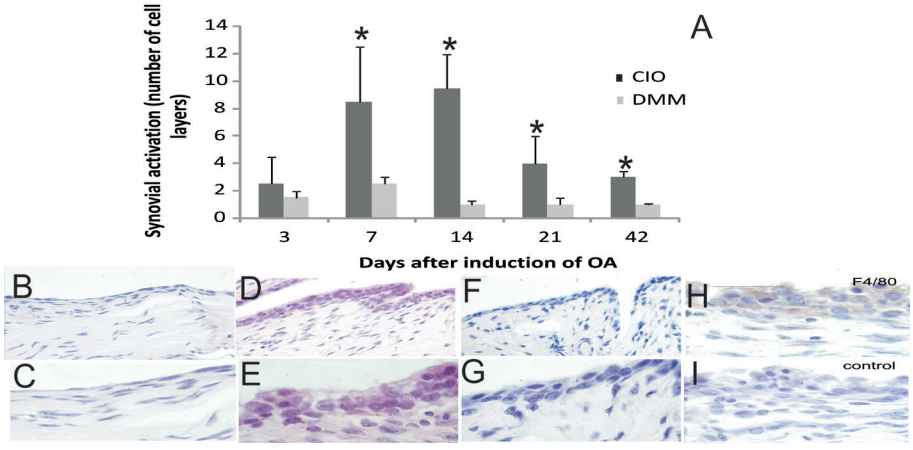


Figure 1: Synovial activation is higher in collagenase-induced osteoarthritis (CIO) when compared to destabilization medial meniscus (DMM) induced murine osteoarthritis. Synovial activation was defined as thickening of the intimal layer. In collagenase-induced osteoarthritis, synovial thickness (3-12 layers: **D** and **E** magnification 250 and 1000X) reached its maximum at day 14 after induction and was significantly higher when compared to the lining thickness observed in DMM (1-3 layers: **F** and **G** magnification 250 and 1000X) (**A**). In normal mouse knee joint, the intima lining layer is only one layer thick and consisting of flattened cells (**B** and **C** magnification 250 and 1000X). In the CIO, the intimal lining comprised large hypertrophic cells which particularly consisted of F4/80 positive macrophages (**H** and **I** control (with relevant first IgG). Data were evaluated using the Mann Whitney U test. Synovial activation CIO versus DMM *P<0.05.

Expression of S100A8 and S100A9 protein in synovium of both OA models was further investigated using immunolocalisation. Strong expression of S100A9 (and in lesser extent S100A8, data not shown) was found in intimal layers of collagenase-induced OA at day 7 (Figure 2C) and was still detectable at day 42 (Figure 2E). No staining of S100A8 or A9 was found in the intimal layer of OA knee joints of S100A9-/- mice at day 7 after induction (Figure 2D). In contrast to the prominent expression within knee joints with collagenase-induced OA, in the DMM model, expression of S100A8 and S100A9 was almost absent within the thin intimal lining layer at day 7 after induction (Figure 2F). Staining of S100A8 and -A9 protein was negative in synovial lining of normal mouse knee joints (data not shown).

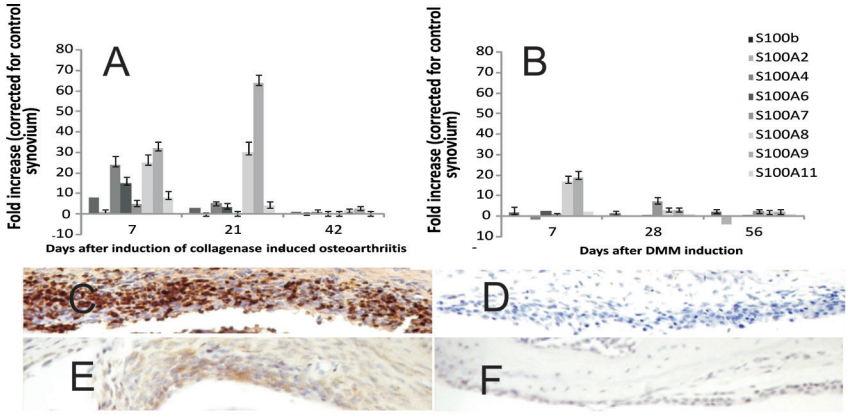


Figure 2: High and prolonged expression of S100A8/A9 during collagenase-induced osteoarthritis (CIO) but not destabilization medial meniscus (DMM) induced osteoarthritis.
Expression of mRNA levels of various members of the S100 family in synovial activation obtained at various time-points after induction of CIO (**A**) or DMM (**B**). Note that mRNA expression of both S100A8 and S100A9 in synovium of CIO remains high up until day 21 whereas other members of the S100 family were low at that time-point. In the synovium of DMM, only S100A8 and S100A9 were high at day 7 but waned thereafter, reflecting the thickness of the lining layer. Values are the mean and SD and were compared to normal synovium. Total knee joint sections were stained with specific antibodies against S100A8 and S100A9. Strong expression of S100A9 (**C**) and S100A8 (not shown) was found in synovial lining at day 7 but also at day 42 although less pronounced (**E**). Staining of lining cells was absent in mice deficient for S100A8/A9 at day 7 after induction of CIO (**D**). S100A9 expression was also absent in the lining of knee joints at day 7 after induction of DMM (**F**). Original magnification of the photomicrographs X400.

S100A8/A9 expression is prolonged during collagenase-induced OA, but not SCW-induced arthritis

Next, expression of S100A8/A9 in the synovium of collagenase-induced osteoarthritis was compared with that in synovium of an acutely inflamed joint. Arthritis was induced by injection of 25 μg of SCW into the mouse knee joint. Total knee joint sections show that shortly after injection, cellularity of the synovium was significantly higher in arthritis when compared to osteoarthritis (data not shown). At day 7 after induction, thickness of the intimal layer was comparable between the two models (Figure 3A-B). In the SCW arthritic knee joint, S100A8/A9 mRNA levels were high shortly after injection but rapidly normalized and were not detected anymore at day 7 after injection (Figure 3C). In contrast, in the OA model, although showing comparable intimal thickness at day 7, S100A8/A9 levels remained high and even increased between 7 and 21 days. This suggests that within this OA model synovial intimal lining cells are activated for prolonged

periods of time, probably through factors released from the affected cartilage during the course of OA.

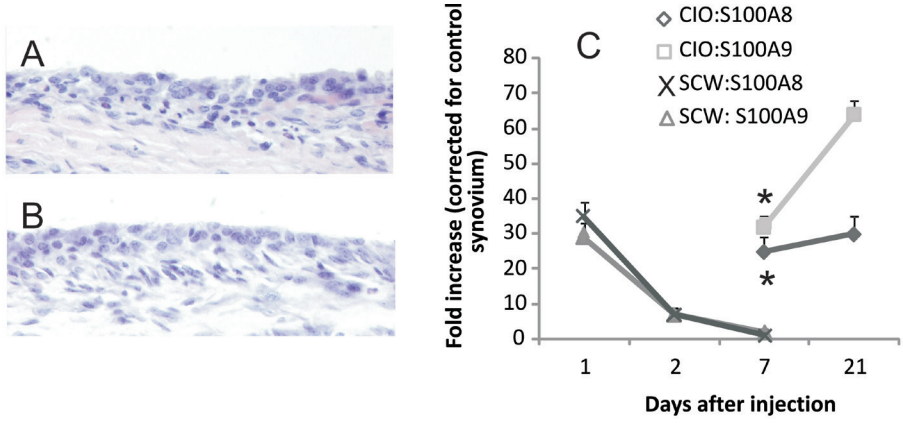


Figure 3: S100A8/S100A9 is tightly regulated in streptococcal cell wall (SCW) induced arthritis but expressed for prolonged periods in collagenase-induced OA (CIO).
Arthritis was induced by a single injection of 25 μg of SCW. Synovial activation was measured in inflamed knee joints as thickening (number of cell layers) of the synovial intimal lining layer. At day 7, synovial thickness in the SCW arthritis was comparable with that seen in CIO and representative photomicrographs are shown in **A** (=SCW arthritis at day 7) and **B** (=CIO at day 7). Note that expression of mRNA levels of S100A8 and S100A9 in the synovium of SCW arthritis was highest at day 1 and at day 7, despite a strongly thickened lining layer, had returned to basic levels. In contrast in synovia of CIO, levels remained high up till day 21 after induction (**C**). Data were evaluated using the Mann Whitney U test (*$P<0.05$). Photomicrograph magnification X 250.

Lack of prolonged expression of IL-1β in collagenase-induced OA

Apart from S100A8/A9, we determined expression of other pro-inflammatory cytokines/chemokines which might be involved in driving synovial activation. Pro-inflammatory cytokines/chemokines were hardly expressed (IL-6, TNFα, MIP1α and KC; 16,11,7 and 5 fold increase respectively) during collagenase-induced osteoarthritis with the exception of IL-1β and its antagonist IL-1ra which were upregulated 65 and 99 times respectively. In contrast to S100A8 and S100A9, expression of IL-1β was already markedly reduced at day 21. (IL-1β between 7-9 times and S100A8 and S100A9 between 30-35 and 66-69 times higher when compared to normal synovium) (Figure. 4A).

S100A8/A9 regulation of synovial activation during collagenase-induced OA

We next investigated the effect of S100A8/A9 in development of synovial lining thickness using S100A9-/- mice. S100A9-/- mice have a normal phenotype whereas S100A8-/- mice are not viable (34). S100A9-/- mice also lack S100A8 protein in their myeloid cells. S100A9-/- is thus functionally a double knockout, probably due to a high turnover of S100A8 in the absence of its binding partner S100A9 (17). When collagenase-induced-OA was elicited in knee joints of S100A9-/- mice, significantly less thickening of the synovial lining was observed when compared to WT mice. Synovial thickness was 62% lower at day 42 when compared to OA induced in WT controls (Figure 4B-C-D).

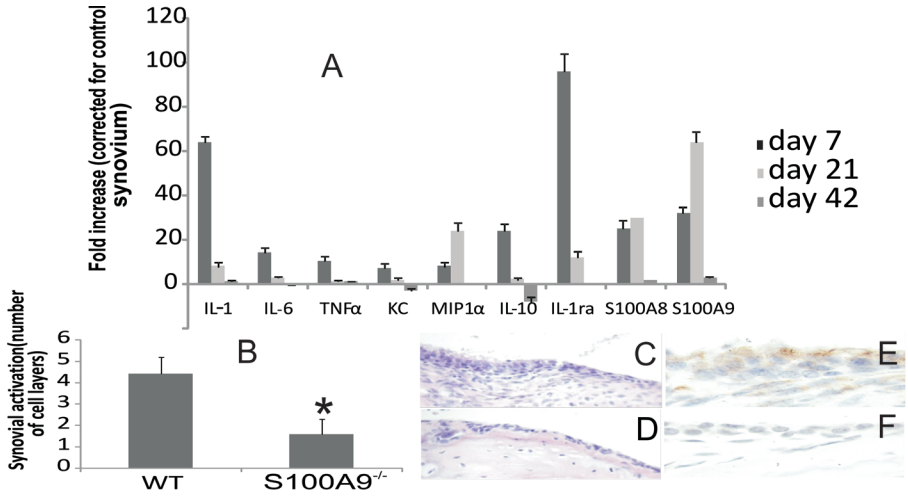


Figure 4: High and prolonged expression of S100A8/A9 and decreased synovial thickening in S100A9 $^{-/-}$ mice during collagenase-induced OA
Expression of mRNA levels of S100A8/S100A9 is higher and more prolonged than pro- and anti-inflammatory cytokines in activated synovium during collagenase-induced osteoarthritis (CIO) when compared to normal synovium (**A**). Note that apart from S100A8 and S100A9 also IL-1 and IL-1ra were highly expressed in the synovium at day 7 after OA induction but strongly decreased thereafter and were low at day 21. Thickness of the synovial lining layer (number of cell layers) was determined in knee joints of S100A9-/- mice and their wild type controls (WT) at day 42 after induction of CIO (**B**). Note that synovial thickness was significantly lower in the OA synovium of S100A9-/- mice (**B** and **C**=day 42 CIO in WT and **D**=day 42 CIO in S100A9-/- mice). Knee joints were stained with anti-NITEGE antibodies which recognize neoepitopes generated by ADAMTS. Clear staining was observed in the lining of day 42 CIO in WT (**E**) which was absent in the synovial lining of the S100A9-/- (**F**). Bars show the mean and SD scores from 15 animals performed in two different experiments. Values were evaluated using the Mann Whitney U test (*P<0.05). Photomicrographs magnification X250 (**D** versus WT **C**) or X1000 (**F** versus WT **E**).

Synovial lining cells express enzymes like ADAMTS which cleave proteoglycans thereby leaving NITEGE neoepitopes. At day 42 after OA induction, synovial intima lining cells stained strongly positive for NITEGE neoepitopes (Figure 4E) whereas no staining was observed within the intimal lining of S100A9-/- mice (Figure 4F). In contrast, in the DMM model, in which only low staining of S100A9 in the synovium was found, only minimal synovial thickness was observed which was not different between S100A9-/- and WT mice (data not shown).

S100A8/A9 determination of cartilage destruction in collagenase-induced, but not destabilized medial meniscus–induced, OA

Subsequently, we explored whether S100A8/A9 mediates severe cartilage destruction during collagenase-induced osteoarthritis using histology. Cartilage destruction was measured using an arbitrary scale developed by Pritzker et al (28) and adapted by us for mice. Cartilage destruction was measured in various cartilage surfaces (medial and lateral femur and tibia) of the knee joint. Cartilage destruction was significantly lower in S100A9-/- in all surfaces (Figure 5A) and ranged from a 45% reduction in the lateral tibia to 73% reduction in the medial femur when compared to OA in WT. (Figure 5B-C). When DMM was induced in knee joints of WT mice, most cartilage matrix destruction at day 56 was observed in the medial tibia and femur whereas less destruction was observed at the lateral side (Figure 5D-E-F). Interestingly, no significant differences were measured between S100A9-/- and WT mice, suggesting that S100A8/A9 do not mediate cartilage destruction within this model.

Elevated expression of S100A8/A9 in synovial biopsy samples from OA patients

In synovial biopsies from early OA patients (n=19), high mRNA levels of S100A8 and A9 were measured when compared to “normal” synovium (obtained during an arthroscopic procedure in patients with suspected acute joint trauma). S100A8 and A9 levels were raised 11 and 10 fold respectively (Figure 6A). Joint pathology in knee joints of CHECK-patients was determined using the Kellgren Lawrence score (KL). Interestingly, levels of S100A8 mRNA were significantly enhanced by 4,5 fold in patients with a KL > 0 at the time of biopsy (Figure 6B) compared to those with a KL=0 ($p=0.046$).

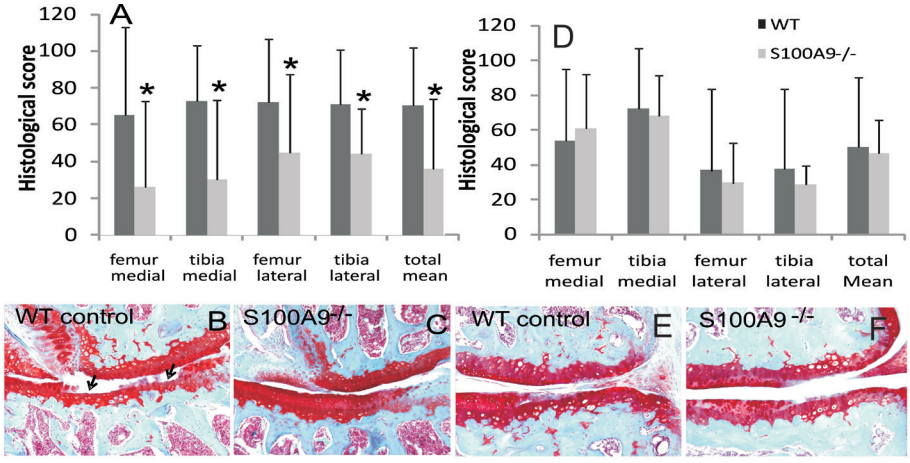
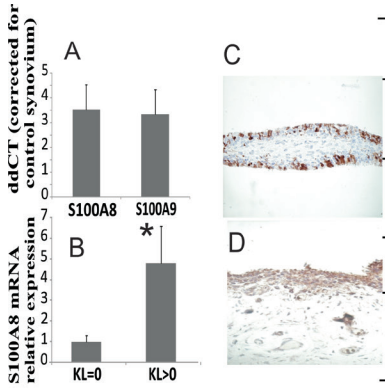


Figure 5: S100A8/S100A9 regulate cartilage destruction during collagenase-induced osteoarthritis (CIO).
Destruction was measured in cartilage layers of medial and lateral tibia and femur of the knee joint at day 42 after induction of CIO or at day 56 after induction of destabilization medial meniscus (DMM) osteoarthritis. In the CIO model, cartilage damage was significantly lower in all cartilage layers of the OA knee joints of S100A9$^{-/-}$ mice (**A** and **C** versus WT control **B**). In the DMM model no significant differences were observed (**D** and **F** versus WT control **E**). Bars show the mean and SD scores of 8 animals per group. Two independent experiments were performed. Values were evaluated using the Mann Whitney U test (*P<0.05). Magnification of the photomicrographs X 250).

Protein expression of S100A8 and S100A9 was determined using immunohistochemistry. S100A8 was abundantly expressed in biopsies of early OA(n=19) (Figure 6C) as well as in synovium from late stage OA(n=10)(Figure 6D). Control irrelevant IgG was negative (data not shown). Similar expression was observed for S100A9 (data not shown) suggesting that these proteins are produced during prolonged periods throughout the osteoarthritic process. High S100A8 levels were particularly found within synovia containing a thickened intima. Positive staining was mainly found within CD68 positive macrophages in the lining layer, subintimal lining layer and surrounding the blood vessels (Figure 6D). The number of S100A8 and S100A9 positive cells varied between patients: 45-60% of total intima cells in early and between 50-60% of total intima cells in late synovial specimen. The intima lining thickness and subintima cellularity were determined and correlated to expression of S100A8 and S100A9 protein. A significant positive correlation was found between S100A8 expression and intima lining thickness and subintima cellularity (Figure 6E) and a similar correlation was found for S100A9.



E

		Spearman R	95% confidence range	p-value	Significance
S100A8	Intima	0,65	0,268-0,857	0,0025	**
	Subintima	0,63	0,231-0,847	0,0039	**
S100A9	Intima	0,51	0,055-0,787	0,027	*
	Subintima	0,49	0,037-0,780	0,032	*

F

	S100A8/9 levels	SEM	p-value	N	m/f	Age	BMI
Non-progressors	487	30,7		95	21/74	57,6	25,5
Progressors	581*	34,8	0,0175	82	15/67	57,4	27,1

Figure 6: S100A8 and S100A9 are highly expressed in biopsies of early and end stage human OA and mRNA levels of S100A8 predict joint damage at year 2.
mRNA levels of both proteins were significantly elevated when compared to "normal" synovium (**A**). High expression of S100A8 correlated significantly with a positive Kellgren Lawrence score (**B**). In the synovial biopsies of early OA patients, both S100A8 (**C**) and S100A9 (data not shown) staining was found which was expressed by macrophages lying in the intima lining layer. Similar stainings were found in synovia of late OA patients (S100A8 staining in **D** and S100A9 (not shown)). Using irrelevant IgG no staining was found (data not shown). Original magnification photomicrographs X400. A significant correlation was found between S100A8/9 protein expression and synovium activation parameters (**E**). Furthermore, S100A8/A9 serum levels predict joint damage in human OA (**F**). Comparison of mean serum S100A8/S100A9 levels in patients without damage at base line, which do (82 patients) or do not show (95 patients) progression of joint damage (K&L) after two years. Data were evaluated using the Mann Whitney U test and significance is indicated by * ($P<0.05$). Confounding by BMI was excluded by multiple regression analysis.

S100A8/A9 prediction of early progression of joint destruction during human OA

To study whether S100A8/A9 levels are associated with progression of joint damage, S100A8/A9 was measured in sera from early symptomatic OA patients (CHECK cohort) at baseline using an ELISA. Joint destruction was determined at baseline and 2 years after the start of the study, using the Kellgren & Lawrence score of radiographs. Two patient groups were defined: [1] Non-progressors and [2] Progressors. No significant differences were found in demographic data like age and BMI between the groups. Strikingly, significantly higher serum levels of S100A8/A9 were found in the group of progressors compared to non-progressors (Figure 6F). The mean serum level in serum of non-progressors was 487 ng/ml (n=95), and in the progressor group 581 ng/ml (n=82), indicating a potential value for serum-levels as predictor for progression.

Discussion

In the present study we find that S100A8/S100A9 are pivotal proteins involved in mediating cartilage destruction during experimental collagenase-induced OA in which synovial activation is important for induction of joint pathology but not in the DMM model in which synovial activation is absent. Arthroscopic studies performed in joints of OA patients suggest that localized proliferation and inflammatory changes in the synovium occur in around 50% of OA patients (7-8). Macrophages are dominant cell types present in activated synovium of human OA although also B and T cells have been described (11). As in human OA, also in collagenase-induced osteoarthritis, macrophages form the predominant cell type in the inflamed synovium and no PMNs are observed during the chronic phase between day 7 and day 42.

S100A8/S100A9 appeared to be important in sustaining synovial activation during collagenase-induced OA as S100A9-/- mice whose peripheral myeloid cells also lack the S100A8 protein (24) show reduced synovial activation and cartilage destruction. Both mRNA as well as protein levels of S100A8 and A9 remained high for extended periods (up to 21 days after injection) in the synovium of collagenase-induced OA. S100A9 mRNA expression even increased between day 7 and day 21. This is in line with our human data which show that both biopsies from patients with early symptomatic as from patients with end stage OA express high levels of S100A8 and A9 protein suggesting a prolonged expression throughout the OA process. S100A8 and A9 have been shown to induce a specific inflammatory response in human microvascular endothelial cells, macrophages as well as osteo- and chondroblasts (35). Synovial activated macrophages are the source of S100A8/S100A9 in collagenase-induced OA.

Apart from regulating synovial inflammation, the macrophage has also been shown to mediate cartilage destruction. In earlier studies we found that removal of synovial macrophages prior to induction of experimental osteoarthritis almost completely blocked metalloproteinase-induced cartilage destruction and osteophyte formation (12,36). Synovial inflammation has been considered as a factor that most likely contributes to dysregulation of chondrocyte function favoring an imbalance between the catabolic and anabolic activities of the chondrocyte in remodeling the cartilage extracellular matrix (37). Earlier studies

showed that intra-articular injection of S100A8 into murine knee joints strongly stimulate both cytokine and MMP/ADAMTS expression in the intima layer (23). Fibroblast-like type B cells lying within the intimal layer produce proteoglycans and at the same time are high producers of ADAMTS (38) which may explain the presence of large amounts of NITEGE neoepitopes within the intimal layer of collagenase-induced OA. In contrast, stimulation of macrophages with S100A8 upregulated only cytokines but not MMP/ADAMTS suggesting that the contribution of macrophages to cartilage destruction may be through abundant release of catabolic cytokines and S100 species thereby further stimulating fibroblast-like type B cells to produce ADAMTS.

IL-1β, IL-6 and TNFα are all capable of inducing MMPs, aggrecanases and other catabolic factors (39). TNFα and IL-1β co-localized with MMP-1,-3,-9 and 13 in regions of matrix depletion in OA cartilage (40). There is a strong relationship between the increased levels of catabolic enzymes and inflammatory mediators like IL-1. IL-1 has earlier been shown to be crucial in mediating cartilage destruction in experimental murine arthritis (41). The role of IL-1 in driving OA pathology is however still a matter of debate (14). We found a two times downregulation of IL-1 in the synovium of the knee joint after a short initial phase of upregulation. IL-1β and its effects are probably inhibited by cytokines like IL-10 and IL-1ra which were highly expressed shortly after OA induction. IL-1β drives cartilage destruction by stimulating MMP production in chondrocytes. However MMPs are secreted in a latent form and need an activation step in order to become activated. S100A8 protein may be involved in this by induction of MMP activating factors like oxygen radicals (23).

Interestingly when pro-inflammatory triggers like SCW fragments were injected into a normal mouse knee joint, only a short-lasting enhancement of S100A8 and S100A9 expression in the synovium was measured indicating that expression of both proteins is tightly controlled during acute inflammation. Expression of S100A8 and S100A9 for prolonged periods in synovium during collagenase-induced OA implies that the expression becomes reactivated, which may be driven by cartilage degradation products released during OA. Interestingly, whereas S100A8 and S100A9 levels remain high up till day 21, expression of cytokines like IL-1,TNF and IL-6 were already strongly decreased at this time-point. Although pathogenic triggers which are responsible for prolonged and specific up-regulation of S100A8/S100A9 in OA are not known, recent data point to a pro-

inflammatory crosstalk between ECM metabolites and phagocyte-specific danger molecules. Elevated catabolic breakdown of the matrix by metalloproteinases leads to additional release of matrix products like byglycan, decorin and aspirin (42). Biglycan has recently been shown to activate the inflammasome within macrophages (43) and may reactivate macrophages forming a positive feedback loop with S100A8/S100A9. Interestingly, both molecules stimulate macrophages via TLR4 (44).

Apart from having an effect on synovial inflammation, S100 protein may have a direct effect on chondrocyte metabolism(23). Although homo- and heterodimers of S100A8 and S100A9 are negatively charged they may penetrate cartilage layers due to their small 20 kD size (45). In a recent study using human chondrocytes isolated from OA cartilage, we found that not only S100A8 but also S100A9 were potent stimulators of a catabolic phenotype reflected by a high release of MMP-1,-3 and-9 and an inhibition of matrix molecules, collagen type II and aggrecan (manuscript in press). MMP-3 is a crucial enzyme involved in cartilage destruction both during experimental arthritis (46) as well as during collagenase-induced osteoarthritis (13).

The mechanism how S100A8 activates chondrocytes is not known. Various receptors have been described to be involved in S100A8 signaling like TLR4 (17,47), RAGE (48) and N-glycans (49). In previous studies, it was shown that S100A8 signaling in macrophages occurs via TLR4 (17). Using primary chondrocytes obtained from arthroplasties of OA patients, we recently found that stimulation by S100A8 and S100A9 was significantly inhibited by the TLR4 inhibitor TAK242 but not by inhibitors of RAGE or N-glycans (manuscript in press). In synovial fluids of OA patients abundant amounts of the S100A8/S100A9 (up to 5-7 μg/ml) have been detected (50). Significantly elevated S100 concentrations, probably spillover of the joints, were also measured in sera of patients suspected from OA when compared to healthy controls. Interestingly significant higher levels were measured in those OA patients at baseline which had developed a clear cartilage destruction 2 years later. When a cut-off was defined at 600 ng/ml, above which the level of S100A8/A9 was defined increased, and only patients with severe progression (delta K&L ≥3) were analyzed, a significantly higher number of patients with severe progression showed increased levels of S100A8/A9 (chi square-test). The odds-ratio was 7.5 indicating a highly increased risk of severe progression when S100A8/A9 levels are increased (results not shown).

However, numbers of strong progressors were very low (n=8) and further research at later follow-up time points are planned to determine the value of these proteins as progression markers.

Synovial activation which is clearly present within a subpopulation of OA patients may explain the high levels of S100A8 and S100A9 found within synovial fluids but also the blood where they can easily be measured. As S100A8 and S100A9 levels remain high for prolonged periods and as they are important stimulators of cartilage destruction, these proteins may be effective biomarkers for predicting progressive cartilage destruction in OA.

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References

1. Goldring MB, Goldring SR. Osteoarthritis. J Cell Physiol. 2007;213:626-34.

2. Blom AB, van der Kraan PM, van den BergWB. Cytokine targeting in osteoarthritis. Curr Drug Targets 2007;8:283-92.

3. van den Berg, WB. Lessons from animal models of osteoarthritis. 2008. Curr Rheumatol Rep. 10(1):26-9.

4. CawstonTE, Young DA. Proteinases involved in matrix turnover during cartilage and bone breakdown. Cell Tissue Res. 2010;339:221-35.

5. Krasnokutsky S, Attur M, Palmer G, Samuels J, Abramson SB. Current concepts in the pathogenesis of osteoarthritis. Osteoarthritis Cartilage 2008;16:S1-3.

6. Abramson SB, Attur M. Developments in the scientific understanding of osteoarthritis. Arthritis Res Ther 2009;11:227.

7. Sellam J, Berenbaum F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nat Rev Rheumatol. 2010;6:625-35.

8. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral kneeosteoarthritis -- results of a 1 year longitudinal arthroscopic study in 422 patients. Osteoarthritis Cartilage 2005;13:361-7.

9. Mazières B, Garnero P, Guéguen A, Abbal M, Berdah L, Lequesne M et al. Molecular markers of cartilage breakdown and synovitis at baseline as predictors of structural progression of hip osteoarthritis. The ECHODIAH Cohort. Ann Rheum Dis 2006;65:354-9.

10. Roemer FW, Guermazi A, Hunter DJ, Niu J, Zhang Y, Englund M et al. The association of meniscal damage with joint effusion in persons without radiographic osteoarthritis: the Framingham and MOST osteoarthritis studies. Osteoarthritis Cartilage 2009;17:748-53.

11. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum. Dis 2005;64:1263-7.

12. Blom AB, van Lent PL, Libregts S, Holthuysen AE, van der Kraan PM et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. Arthritis Rheum 2007;56:147-57.

13. Bondeson J, Blom AB, Wainwright S, Hughes C, Caterson B, van den Berg WB. The Role of synovial macrophages and macrophage- produced mediators in driving inflammatory and destructive responses in osteoarthritis. Arthritis Rheum 2010;62:647-57.

14. Heizmann CW, Fritz G, Schäfer BW. S100 proteins: structure, functions and pathology. Front Biosci 2002;7:1356-68.

15. Foell, D, Wittkowski H, Roth J. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. Nat. Clin. Pract. Rheumatol 2007;3:382-90.

16. Vogl T, Ludwig S, Goebeler M, Strey A, Thorey IS, Reichelt R et al. MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. Blood 2004;104:4260-8.

17. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 2007;13:1042-9.

18. Ehrchen JM, Sunderkötter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection,autoimmunity, and cancer. J Leukoc Biol. 2009; 86:557-66.

19. Chen YS, Yan W, Geczy CL, Brown MA, Thomas R.Serum levels of soluble receptor for advanced glycation end products and of S100 proteins are associated with inflammatory, autoantibody, and classical risk markers of joint and vascular damage in rheumatoid arthritis. Arthritis ResTher. 2009;11:R39.

20. Hammer HB, Odegard S, Syversen SW, Landewé R, van der Heijde D, Uhlig T et al. Calprotectin (a major S100 leucocyte protein) predicts 10-year radiographic progression in patients with rheumatoid arthritis. Ann.Rheum.Dis 2010;69:150-4.

21. van Lent PL, Grevers L, Blom AB, Sloetjes A, Mort JS, Vogl T et al. Myeloid-related proteins S100A8/S100A9 regulate joint inflammation and cartilage destruction during antigen-induced arthritis. Ann Rheum Dis 2008;67:1750-8.

22. Zreiqat H, Belluoccio D, Smith MM, Wilson R, Rowley LA, Jones K, et al. S100A8 and S100A9 in experimental osteoarthritis. Arthritis Res Ther 2010;12:R16.

23. van Lent PL, Grevers LC, Blom AB, Arntz OJ, van de Loo FA, van der Kraan P et al. Stimulation of chondrocyte-mediated cartilage destruction by S100A8 in experimental murine arthritis. Arthritis Rheum 2008;58:3776-87.

24. Manitz MP, Horst B, Seeliger S, Strey A, Skryabin BV, Gunzer M et al. Loss of S100A9 (MRP14) results in reduced interleukin-8-induced CD11b surface expression, a polarized microfilament system, and diminished responsiveness to chemoattractants in vitro. Mol Cell Biol 2003;23:1034-43.

25. van der Kraan PM, Vitters EL, van Beuningen HM, van de Putte LB, van den Berg WB. Degenerative knee joint lesions in mice after a single intra-articular collagenase injection. A new model of osteoarthritis. J Exp Pathol 1990;71:19-31.

26. Ma HL, Blanchet TJ, Peluso D, Hopkins B, Morris EA, Glasson SS.Osteoarthritis severity is sex dependent in a surgical mouse model. Osteoarthritis Cartilage 2007;15:695-700.

27. Geurts J, Vermeij EA, Pohlers D, Arntz OJ, Kinne RW, van den Berg WB et al. A novel Saa3-promoter reporter distinguishes inflammatory subtypes in experimental arthritis and human synovial fibroblasts. Ann Rheum Dis 2011;70:1311-9.

28. Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA et al. Osteoarthritis cartilage histopathology: grading and staging. Osteoarthritis Cartilage. 2006;14:13-29.

29. Van Meurs JB, Van Lent PL, Joosten LA, Van der Kraan PM, Van den Berg WB. Quantification of mRNA levels in joint capsule and articular cartilage of the murine knee joint by RT-PCR: kinetics of stromelysin and IL-1 mRNA levels during arthritis. Rheumatol Int 1997;16:197-205.

30. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheum1986;29:1039-49.

31. Wesseling J, Dekker J, van den Berg WB, Bierma-Zeinstra SM, Boers M, Cats HA et al. CHECK (Cohort Hip and Cohort Knee): similarities and differences with the Osteoarthritis Initiative. Ann Rheum Dis 2009;68:1413-9.

32. Wulffraat NM, Haas PJ, Frosch M, De Kleer IM, Vogl T, Brinkman DM et al. Myeloid related protein 8 and 14 secretion reflects phagocyte activation and correlates with disease activity in juvenile idiopathic arthritis treated with autologous stem cell transplantation. Ann Rheum Dis 2003;62:236-41.

33. Pessler F, Dai L, Diaz-Torne C, Gomez-Vaquero C, Paessler ME, Zheng DH et al. The synovitis of "non-inflammatory" orthopaedic arthropathies: a quantitative histological and immunohistochemical analysis. Ann Rheum Dis 2008;67:1184-7.

34. Passey RJ, Williams E, Lichanska AM, Wells C, Hu S, Geczy CL et al. A null mutation in the inflammation-associated S100 protein S100A8 causes early resorption of the mouse

embryo. J Immunol 1999;163:2209-16.

35. Viemann D, Strey A, Janning A, Jurk K, Klimmek K, Vogl T et al. Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. Blood 2005;105:2955-62.

36. van Lent PL, Blom AB, van der Kraan P, Holthuysen AE, Vitters E, van Rooijen N et al. Crucial role of synovial lining macrophages in the promotion of transforming growth factor beta-mediated osteophyte formation. Arthritis Rheum 2004; 50:103-11.

37. Loeser RF Molecular mechanisms of cartilage destruction: mechanics, inflammatory mediators, and aging collide. Arthritis Rheum 2006; 54:1357-60.

38. Vankemmelbeke MN, Holen I, Wilson AG, Ilic MZ, Handley CJ, Kelner GS, et al. Expression and activity of ADAMTS-5 in synovium. Eur J Biochem 2001;268:1259-68.

39. Tetlow LC, Adlam DJ, Woolley DE. Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 2001;44:585-94.

40. Wu W, Billinghurst RC, Pidoux I, Antoniou J, Zukor D, Tanzer M et al. Sites of collagenase cleavage and denaturation of type II collagen in aging and osteoarthritic articular cartilage and their relationship to the distribution of matrix metalloproteinase 1 and matrix metalloproteinase 13. Arthritis Rheum 2002;46:2087-94.

41. Dinarello CA Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood 2011;117:3720-32.

42. Zhen EY, Brittain IJ, Laska DA, Mitchell PG, Sumer EU, Karsdal MA et al. Characterization of metalloprotease cleavage products of human articular cartilage. Arthritis Rheum 2008;58:2420-31

43. Babelova A, Moreth K, Tsalastra-Greul W, Zeng-Brouwers J, Eickelberg O, Young MF et al. Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors. J Biol Chem 2009;284:24035-48.

44. Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M et al. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. J Clin Invest 2005;115:2223-33.

45. van Lent PL, van den Berg WB, Schalkwijk J, van de Putte LB, van den Bersselaar L. The impact of protein size and charge on its retention in articular cartilage. J Rheumatol 1987;14:798-805.

46. van Meurs J, van Lent P, Holthuysen A, Lambrou D, Bayne E, Singer I et al. Active matrix metalloproteinases are present in cartilage during immune complex-mediated arthritis: a pivotal role for stromelysin-1 in cartilage destruction. J Immunol 1999;163:5633-9.

47. van Lent PL, Grevers LC, Schelbergen R, Blom A, Geurts J, Sloetjes A et al. S100A8 causes a shift toward expression of activatory Fcγ receptors on macrophages via toll-like receptor 4 and regulates Fcγ receptor expression in synovium during chronic experimental arthritis. Arthritis Rheum 2010;62:3353-64.

48. Gebhardt C, Riehl A, Durchdewald M, Németh J, Fürstenberger G, Müller-Decker K et al.RAGE signaling sustains inflammation and promotes tumor development.J Exp Med 2008; 205:275-85.

49. Turovskaya O, Foell D, Sinha P, Vogl T, Newlin R, Nayak J et al. RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis.Carcinogenesis 2008; 29:2035-43.

50. Sunahori K, Yamamura M, Yamana J, Takasugi K, Kawashima M, Yamamoto H et al. The S100A8/A9 heterodimer amplifies proinflammatory cytokine production by macrophages via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase in rheumatoid arthritis.Arthritis Res Ther 2006;8:R69.

Chapter 3

Alarmins S100A8/S100A9 aggravate osteophyte formation in experimental osteoarthritis and predict osteophyte progression in early human symptomatic osteoarthritis

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Chapter 3

Alarmins S100A8/S100A9 aggravate osteophyte formation in experimental osteoarthritis and predict osteophyte progression in early human symptomatic osteoarthritis

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Abstract

Objective: Alarmins S100A8 and S100A9 are major products of activated macrophages regulating cartilage damage and synovial activation during murine and human osteoarthritis (OA). In the current study we investigated whether S100A8 and S100A9 are involved in osteophyte formation during experimental OA and if S100A8/A9 predicts osteophyte progression in early human OA.

Methods: OA was elicited in S100A9 -/- mice in two experimental models that differ in degree of synovial activation. Osteophyte size, S100A8, S100A9 and VDIPEN was measured histologically. Chondrogenesis was induced in murine mesenchymal stem cells in the presence of S100A8. Levels of S100A8/A9 were determined in plasma of early symptomatic OA participants of the CHECK cohort study and osteophytes measured after two and five years.

Results: Osteophyte size was drastically reduced in S100A9 -/- mice in ligaments and at medial femur and tibia on day 21 and 42 of collagenase-induced OA, in which synovial activation is high. In contrast, osteophyte size was not reduced in S100A9 -/- mice during destabilized medial meniscus OA, in which synovial activation is scant. S100A8 increased expression and activation of MMPs during micromass chondrogenesis, thereby possibly increasing cartilage matrix remodeling allowing for larger osteophytes. Interestingly, early symptomatic OA participants of the CHECK study with osteophyte progression after two and five years had elevated S100A8/A9 plasma levels at baseline, while CRP, ESR and COMP were not elevated at baseline.

Conclusion: S100A8/A9 aggravate osteophyte formation in experimental OA with high synovial activation and may be used to predict osteophyte progression in early symptomatic human OA.

Introduction

Recently the view on osteoarthritis (OA) is shifting from a disease of only the cartilage to a whole joint disease. Synovial activation and pro-inflammatory mediators from the synovium play an important role in the pathogenesis of OA (1-4). An arthroscopic study suggests that synovial activation occurs in 50% of OA patients (5) and synovitis is associated and can predict cartilage pathology (6, 7). We previously showed that synovial macrophages are essential in regulating synovial inflammation and subsequent cartilage erosion during collagenase-induced OA (CIOA) (8). Important products from activated macrophages are the alarmins S100A8 and S100A9. These damage associated molecular patterns bind calcium and can also be released in huge amounts by neutrophils and monocytes (9, 10). S100A8/A9 is highly present in synovial fluid of both rheumatoid arthritis (RA) and OA patients (11) and in RA both serum and synovial fluid levels of S100A8/A9 correlate with radiological damage and disease activity (12, 13). We recently showed that S100A8 and S100A9 induce a catabolic phenotype in human OA chondrocytes by upregulating pro-inflammatory cytokines and MMP-1, -3, -9 and -13 via TLR-4 (14). Furthermore, we demonstrated that S100A9 -/- mice showed reduced cartilage damage and synovial activation during CIOA, but not in the surgically induced DMM (destabilized medial meniscus) OA (15). CIOA has considerably higher synovial activation compared to DMM, indicating the importance of S100A8/A9 in inflammatory OA.

Osteophytes, important hallmarks of OA, are bony outgrowths lined by cartilage that limit joint movement and cause pain. While classic osteophytes originate from the periosteum, similar structures can also develop in ligaments and tendons, further also called osteophytes. Osteophytosis is initiated by mesenchymal stem cells (MSCs) that undergo condensation, chondrogenic differentiation and proliferation. Finally, the chondrocytes go into hypertrophy and blood vessels are formed which give access to osteoblasts and osteoclasts that transforms the structure into bone in a process highly resembling endochondral ossification (16). Earlier, our lab has shown that overexpression of both TGFβ1 and BMP-2 induces osteophyte formation in the mouse knee joint, albeit with different dynamics (17, 18). Consequently, we have shown that osteophyte formation is highly dependent on synovial macrophages, potent producers of TGFβ-

superfamily members (19).

In the current study, we aimed to unravel the role of S100A8/A9 in osteophyte formation in experimental OA models, focusing on the involvement of the synovium. Moreover, we investigated whether S100A8/A9 plasma levels predict osteophyte progression in participants of the Dutch CHECK cohort of early symptomatic OA.

Material and Methods

Animals and experimental OA models

Experimental OA was elicited in 12-14 weeks old male C57BL/6J (Janvier) and S100A9 -/- mice backcrossed to the C57BL/6 background for 12 generations. Mice were housed in groups of 10 animals in filter-top cages, and water and food were provided ad libitum.

Collagenase-induced osteoarthritis (CIOA) was performed by 2 times intra-articular injection of 1U of bacterial collagenase (Sigma-Aldrich), causing disruption of the ligaments and local instability of the knee joint (20). Destabilized medial meniscus (DMM) OA was induced by transection of the medial anterior meniscotibial ligament (21).

Histology

Total knee joints or micromasses were fixed in formalin and cut in 7 or 5 μm sections, respectively. Staining was done with Safranin-O (0.1% in water), counterstaining with Fast Green (0.1% in water). Osteophyte size was assessed in Safranin-O sections using the Leica Application Suite (LAS) image analysis software. Knee joint sections were also immunostained with anti-rabbit S100A8, S100A9 (own facilities) or VDIPEN, with rabbit IgG as isotype. Biotinylated horseradish peroxidase–conjugated goat antirabbit IgG (Dako) was used as secondary antibody, followed by avidin-streptavidin-peroxidase (Elite kit; Vector) and diaminobenzidine to develop peroxidase staining, enhanced with nickel for the VDIPEN staining. Counterstaining was done with Mayer's hematoxylin

(Merck) for S100A8 and S100A9 and with Orange G (2%) for VDIPEN.

Micromass chondrogenesis

Chondrogenesis was studied by bringing the MSC cell line C3H10T1/2 in micromass pellets. 250.000 Cells were allowed to adhere in round-bottom 96-well plates for 3 hours after which they spontaneously form microspheres of cells. Micromasses were stimulated with chondrogenic medium (DMEM with 50µg/ml vitamin C, 6.25µg/ml transferrin, 6.25ng/ml sodium selenite, 5.35µg/ml linoleic acid, 10-7M dexamethasone, 6.25µg/ml insulin, 1.0mg/ml sodium pyruvate, 0.4mg/ml prolin, 2% BSA and 48 µg/ml gentamycin) two times a week for three weeks, supplemented with 5 ng/ml TGFβ1, 50 ng/ml BMP-2 and 5µg/ml recombinant murine S100A8.

Determination of mRNA expression with RT-qPCR

RNA was isolated from micromass cultures using TRI-reagent (Sigma-Aldrich), as described previously (22). RNA from murine synovial biopsies was isolated using the RNeasy-kit (Qiagen), after first disrupting synovial tissue with the MagNA Lyser (Roche). RNA was reverse transcribed to cDNA and RT-qPCR was performed with specific primers and the SYBR Green Master mix in the StepOnePlus real-time PCR system (Applied Biosystems) as described before (22). Expression levels are expressed as (-) delta Ct (dCt) values, normalized to the reference gene GAPDH. Primer-sequences can be found in the supplemental methods.

Measurements in early symptomatic OA cohort (CHECK)

Samples were obtained from the Cohort Heup and Cohort Knie (CHECK). On entry into the CHECK study (=baseline), all participants had pain or stiffness of the knee or hip, and all of them were aged between 45–65 years. They had not yet consulted their physician for these symptoms, or the first consultation occurred within 6 months before cohort entry. Participants with any other pathologic condition that could explain the symptoms were excluded. Right or left knee pain was assessed at baseline and S100A8/A9 was measured in plasma of 58 participants of the CHECK cohort. Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and serum cartilage oligomeric matrix protein (COMP)

was measured in the same participants, as described before (23). Osteophytes of the tibiofemoral joints were made at baseline and after 2 and 5 years by a weight-bearing posteroanterior view, semi-flexed (7–10 degrees). Femoral medial and lateral osteophytes, and tibial medial and lateral osteophytes were scored on a 0-3 scale according to Altman et al. (24). Participants were categorized into osteophyte progressors and non-progressors: Progressors (n=34) were participants that had pain in left or right knee (or both) at baseline and consequently developed osteophytes or increased their osteophyte size after either 2 or 5 years in that particular knee on any of the 4 measured locations. Non-progressors (n=24) did have pain in the knee, but did not develop or increase osteophytes in that particular knee. All except two non-progressors also did not have osteophyte progression in the other knee. More information on the CHECK cohort can be found in the supplemental methods.

Additional methods

Methods on CAGA-luc (TGFβ-activity), S100A8/A9 ELISA as well as information on statistical analysis and ethics can be found in the supplement.

Results

Development of osteophytes during collagenase induced OA coincides with high synovial and serum S100A8/A9 protein levels

Recently, we have shown that S100A8/A9 drives cartilage destruction in collagenase-induced OA (CIOA) (15), a model with high synovial activation. Within this model we explored whether S100A8/A9 is associated with osteophyte formation. On day 7, 21 and 42 after induction of CIOA, we stained whole knee sections with Safranin-O (SafO) and immunostained for S100A8 and S100A9.

After one week of CIOA, osteophytes started to develop, as evidenced by chondrogenesis particularly at the medial and lateral margins of the femur. However, no chondrogenesis was seen in the ligaments yet. Interestingly, very strong S100A9 expression at this time point was found in the synovial lining layer (Figure 1A). On day 21 of CIOA, bone deposition was observed in

the osteophytes as they increased in size. Furthermore, osteophytes started developing in the medial collateral ligament. S100A9 expression on day 21 was still high in the synovial lining layer (Figure 1B). On day 42, fully developed and large osteophytes were observed particularly at the medial side, but also at the lateral side. Although decreased compared to day 7 and 21, S100A9 was still expressed in the synovial lining layer on day 42 (Figure 1C). A naïve knee joint did not show any osteophytes and isotype staining for S100A9 was negative (Figure 1E). Immunostaining of S100A8 was comparable to S100A9 (not shown). S100A8/A9 protein levels in serum were high on both day 7 and day 21 (Figure 1D), and returned to control (non-OA) levels on day 42. Together, these results show that S100A8 and S100A9 are present during CIOA in the synovium during the first phases of osteophytosis.

Osteophyte size is drastically reduced in S100A9 -/- mice with collagenase induced OA

Next, we investigated whether S100A8/S100A9 could affect osteophyte development by inducing CIOA in knee joints of mice deficient for S100A9. The peripheral myeloid cells of these mice also lack S100A8 protein, while S100A8 protein was also hardly detectable in the joint (see supplementary Figure S1), which make them a functional double knockout (25). On day 7, both WT and S100A9 -/- mice showed small areas with chondrogenesis at the medial femur (Figure 2A). Interestingly, on day 21 osteophytes were greatly reduced in S100A9 -/- mice on the margin of the medial femur and tibia (56% and 67% reduction compared to WT, respectively) (Figure 2B) and even slightly more reduced on day 42 (65% and 68% reduction) (Figure 2C-D). Osteophytes at the cruciate ligament were also significantly smaller at day 21 (58% reduction) and a non-significant reduction was observed in the medial collateral ligament (Figure 2B). Finally, we observed a dramatic reduction in osteophyte size at the medial collateral ligament on day 42 in S100A9 -/- mice (92% compared to WT) (Figure 2C-D).

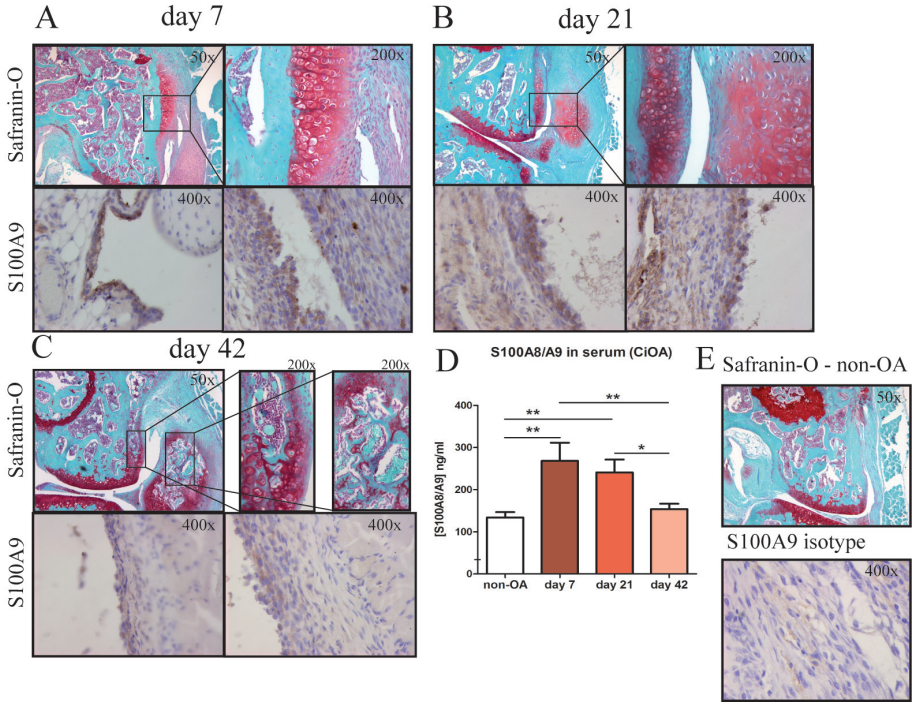

Figure 1: Time course of osteophyte development during collagenase induced OA (CIOA) and S100A8/A9 protein expression in synovium and serum.
CIOA was elicited in C57/Bl6 mice and histology of total knees was taken on 7 (**A**), 21 (**B**) and 42 days (**C**) after induction. At the same days after CIOA induction, S100A8/A9 serum levels were measured with a specific ELISA (**D**). Paraffin-embedded sections were stained for proteoglycans with Safranin-O (**A**,**B**,**C** upper panels) and S100A8 (not shown) and S100A9 were immunostained in adjoining sections (**A**,**B**,**C** lower panels). **A**, On day 7, chondrogenesis in developing osteophytes was almost exclusively seen at the medial and lateral margins of the femur, while S100A9 expression was highly expressed in the synovial lining. **B**, On day 21, chondrogenesis was observed in medial collateral and cruciate ligaments, and at the margins of tibia and femur while S100A9 staining was observed in the synovial lining layer with similar intensity as on day 7.**C**, On day 42, bone development in about 50% of osteophytes in both ligaments and femur and tibia margins was observed, as osteophytes grew to larger size. Interestingly, S100A9 staining was still present in the synovial lining layer, although less intense. **E** Safranin-O staining of naive, non-OA mouse knee joint, showing no chondrogenesis/osteophyte formation. Specific RbIgG isotype staining of S100A9 in the right panel. Magnifications are either 50x or 200x for safranin-O and 400x for S100A9 stained sections. **A**,**B**,**C** n=10 mice/group, results are representative for 2 independent experiments. In **D**, average serum S100A8/A9 levels are shown of 4 independent experiments.

Osteophyte size is not reduced in S100A9 -/- during experimental OA with low synovial activation

To investigate whether S100A8/A9 released by the activated synovium is important in the reduction of osteophyte size, we induced experimental OA in

S100A9 -/- mice by destabilization of the medial meniscus (DMM). In contrast to CIOA, synovial activation is very low during DMM (15). No significant changes in osteophyte size were found on day 56 of DMM, neither at the medial femur nor tibia, the locations to which osteophyte formation is mainly restricted to during DMM (Figure 3A-B). The results in the CIOA and DMM model suggest that S100A8/A9 from the synovium stimulates osteophyte development.

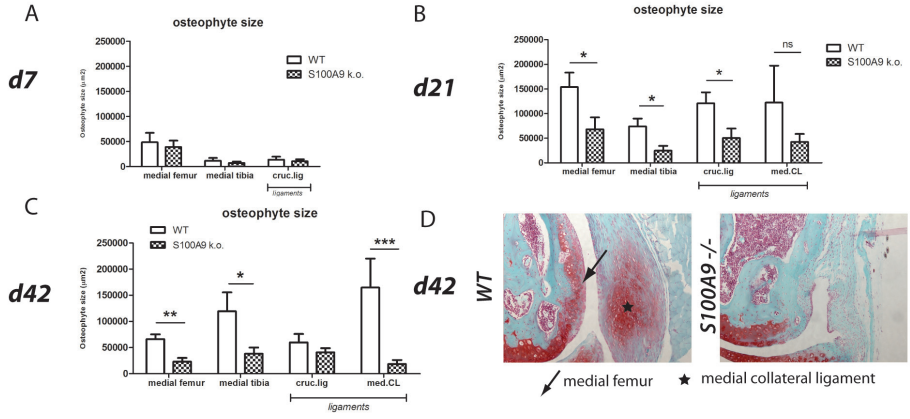

Figure 2: Osteophyte size is drastically reduced in S100A9 -/- mice at day 21 and 42 of collagenase induced-OA
CIOA was elicited in S100A9 -/- mice and osteophyte size was measured with image analysis software at 7 (**A**), 21 (**B**) and 42 (**C**,**D**) days at lateral femur, medial femur and tibia and at medial and lateral collateral ligaments on Saf-O stained histological sections. **A** On day 7, osteophyte size was still small and only slightly reduced at the lateral femur, not on other locations. No chondrogenesis was yet observed inside the lateral and medial collateral ligaments. **B** On day 21, osteophytes were larger and significantly reduced in the S100A9 -/- mice at medial femur and tibia, as well as inside the cruciate ligament. A trend towards smaller osteophyte size was observed inside the medial collateral ligament . **C** On day 42, osteophyte size was drastically reduced at again medial femur and tibia, as well as at the medial collateral ligament. **D** Photomicrographs (magnification 50x) of representative Saf-O stained sections of osteohpytes on day 42 of both WT and S100A9 -/- mice.

S100A8/A9 does not regulate growth factor levels and activation during collagenase induced OA

Earlier, our lab has shown that growth factors such as BMP-2 and TGFβ play a major role in the development of osteophytes during CIOA (17, 26). To investigate whether the reduced osteophyte size in S100A9 -/- mice during CIOA was due to a reduction in growth factors, we measured synovial mRNA expression on 7, 21 and 42 days after induction of OA. No differences in mRNA expression for TGFβ-

family members TGFβ1, BMP-2 and BMP-6 were found between S100A9 -/- and WT (Figure 4A). Nor was there an effect on the activation of TGFβ, as measured with CAGA-luc in synovial washouts (Figure 4B).

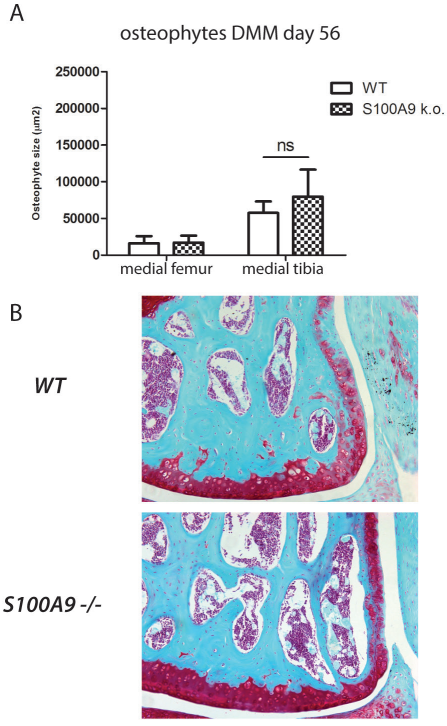

Figure 3: Osteophyte size is not reduced in S100A9 -/- mice with surgically induced DMM OA
Destabilized medial meniscus (DMM) OA was induced in S100A9 -/- mice and osteophyte size was measured on day 56. Osteophytes in the DMM model, which lacks synovial activation, develop almost exclusively at the margins of the medial tibia and femur. **A**, On day 56, osteophyte size at medial tibia and femur was unchanged in S100A9 -/- compared to WT. **B**, Photomicrographs (magnification 50x) of representative Saf-O stained sections of osteohpytes on day 56 of both WT and S100A9 -/- mice.

S100A8/A9 increase remodeling of the cartilage matrix during chondrogenesis by upregulating and activating MMPs

Chondrogenesis of MSCs is an early key feature of osteophyte development (16) and since S100A8/A9 expression was highest at early phases of CIOA (Figure 1), we studied the effect of S100A8 on chondrogenesis in more detail. Chondrogenesis was present in S100A8-supplemented micromasses, as shown by Saf-O staining of proteoglycans. However, the integrity of the S100A8-supplemented micromasses was completely lost as they seemed to collapse and protrusions appeared (Figure 5A). Measuring MMP mRNA levels in these micromasses, we found that particularly MMP-3 was greatly upregulated by adding S100A8 (96-fold), while chondrogenic markers collagen type II and aggrecan were not changed (Figure 5B). Moreover, we also found higher MMP activity as measured by an increase

in the neoepitope VDIPEN (27), which is specific for proteoglycans cleaved by MMPs (Figure 5C). These results indicate that S100A8 can activate MMPs during chondrogenesis. Finally, we analyzed MMP activation during chondrogenesis in vivo, at day 7 of CIOA. High expression of VDIPEN coincided with areas of high proteoglycan content in areas of emerging osteophytes (Figure 5D), suggesting that indeed MMP activation and activity is important in chondrogenesis during osteophyte formation.

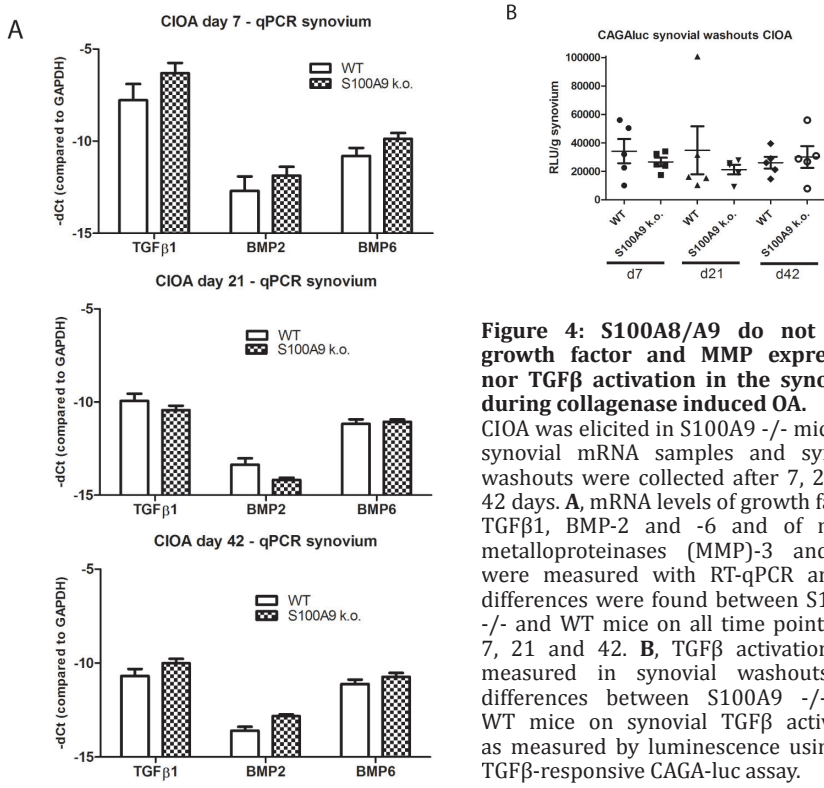

Figure 4: S100A8/A9 do not alter growth factor and MMP expression nor TGFβ activation in the synovium during collagenase induced OA. CIOA was elicited in S100A9 -/- mice and synovial mRNA samples and synovial washouts were collected after 7, 21 and 42 days. **A**, mRNA levels of growth factors TGFβ1, BMP-2 and -6 and of matrix metalloproteinases (MMP)-3 and -13 were measured with RT-qPCR and no differences were found between S100A9 -/- and WT mice on all time points, day 7, 21 and 42. **B**, TGFβ activation was measured in synovial washouts. No differences between S100A9 -/- and WT mice on synovial TGFβ activation as measured by luminescence using the TGFβ-responsive CAGA-luc assay.

S100A8/A9 predicts osteophyte progression in early human symptomatic OA

Our current findings in the mouse prompted us to investigate whether S100A8/A9 levels could predict the progression of osteophytes in humans. For this we measured S100A8/A9 levels in plasma of participants in a sample of the CHECK cohort (n=58) of early symptomatic OA at baseline and scored osteophytes by

radiograph analysis at baseline and after 2 and 5 years (24). Progressors were defined as participants that had increased osteophyte score after 2 or 5 years after having had pain in that knee at baseline, while non-progressors did not increase their knee osteophyte score after 2 or 5 years, despite having knee pain at baseline. Progressors and non-progressors were matched for sex, age and body mass index.

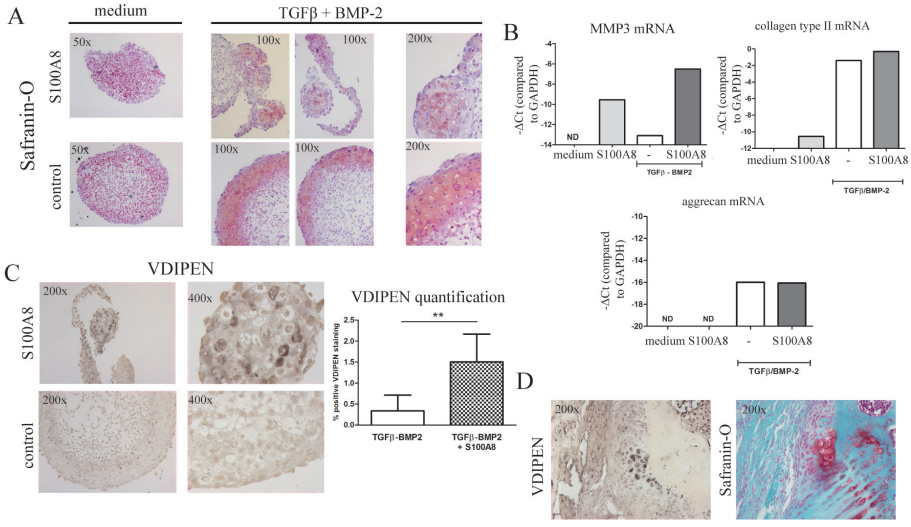

Figure 5: S100A8 increases cartilage remodeling during micromass chondrogenesis by upregulating and activating MMPs.
Chondrogenesis was induced in micromasses of murine mesenchymal stem cells (C3H10T1/2), in the presence of 5 ng/ml TGFβ1 and 50 ng/ml BMP-2, with or without (=control) 5 µg/ml recombinant S100A8. **A**, No chondrogenesis was observed after 21 days in conditions without TGFβ1/BMP-2 (**A**, left panels). Proper chondrogenesis was seen in the TGFβ1/BMP-2 stimulated micromasses as evidenced by Saf-O proteoglycan staining (**A**, bottom middle panels). S100A8 supplemented micromasses did show chondrogenesis, but showed protrusions or total collapse of micromasses (**A**, top middle panels). Detailed pictures (200x magnification) of chondrogenic area with/without S100A8 in top and bottom right panels. **B**, MMP-3, collagen type II and aggrecan mRNA levels were upregulated in micromasses stimulated with S100A8, with or without TGFβ1/BMP-2. **C**, MMP activation was upregulated in S100A8 stimulated micromasses, as evidenced by heightened immunostaining of the MMP-specific cartilage matrix breakdown neoepitope VDIPEN. **D**, On day 7 of CIOA, VDIPEN expression as measure of MMP activation (left panel) coincides with proteoglycan content (Safranin-O, right panel) in developing osteophytes.

Demographic data shows no significant differences between progressors and non-progressors (supplementary Table S1). Interestingly, progressors already had elevated osteophyte scores at baseline (Table S1). Remarkably, we found significantly elevated S100A8/A9 levels in progressors (n=34) of osteophyte formation compared to non-progressors (n=24) (451 ng/ml vs. 254 ng/ml)

(Figure 6A). Furthermore, participants with S100A8/A9 plasma concentrations higher than 200 ng/ml had increased odds of developing osteophytes (odds ratio 4.0; p=0.01, chi square), indicating an increased risk of osteophyte progression when S100A8/A9 levels are high. Moreover, we measured C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) in these participants, common parameters for inflammation in arthritic disease, and found that this was not different between progressors and non-progressors (Figure 6B-C). Also cartilage oligomeric matrix protein (COMP), a widely used marker for cartilage damage, was not changed (Figure 6D).

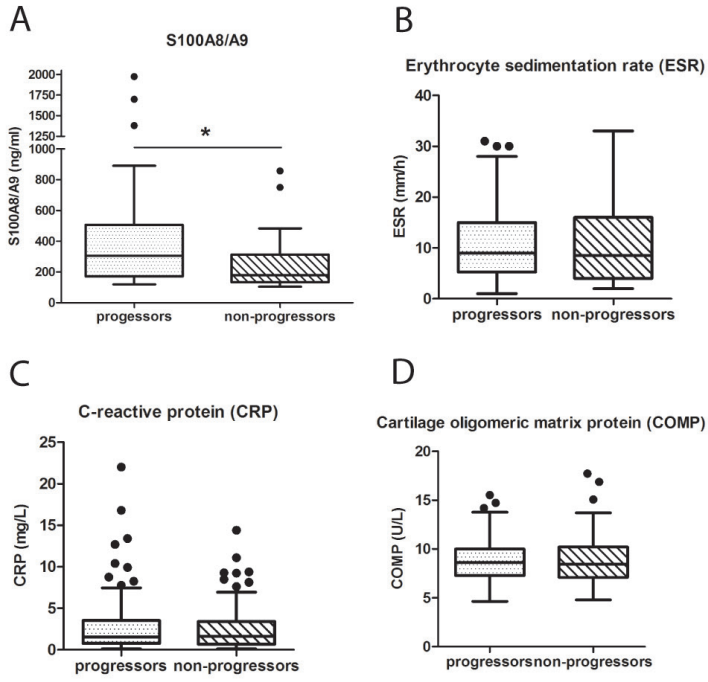

Figure 6: High plasma levels of S100A8/A9 predict knee osteophyte progression at 2 or 5 years in an early symptomatic OA cohort.
Plasma levels of S100A8/A9, serum levels of C-reactive protein (CRP) and cartilage oligomeric matrix protein (COMP), and erythrocyte sedimentation rate (ESR) were measured at baseline in 58 participants of the CHECK cohort. Osteophyte progression was measured by radiograph analysis. **A**, S100A8/A9 plasma levels are significantly upregulated at baseline in participants that developed knee osteophytes after 2 or 5 years after having had knee pain at baseline (=progressors, n=34) compared to participants that did not develop any knee osteophytes after 2 or 5 years, despite having knee pain at baseline (=non-progressors, n=24) **B**,**C** In contrast, standard markers for inflammation, ESR (**B**) and CRP (**C**) are not elevated at baseline in progressors compared to non-progressors. **D** Finally, COMP, a standard clinical marker for cartilage damage, was also not elevated in progressors at baseline.

Discussion

In the current study we show that alarmins S100A8 and S100A9 aggravate osteophyte formation during experimental OA with high synovial activation. Moreover, S100A8/A9 can upregulate and activate MMPs thereby possibly enhancing remodeling during osteophytosis and allowing for larger osteophytes. Finally, we confirmed the relevance of S100A8/A9 during osteophyte formation in humans by showing that elevated S100A8/A9 plasma levels in people with early symptomatic OA at baseline have a predictive value for development of osteophytes after two or five years.

Using S100A9 -/- mice first in CIOA, we have shown that S100A8 and S100A9 are important for the development of large osteophytes at both the bone margins as well as in ligaments. Since we have shown earlier that cartilage damage is reduced in S100A9 -/- mice during CIOA (15), S100A8/A9 could indirectly contribute to osteophyte size through cartilage damage and cartilage breakdown products. However, we clearly show that osteophyte formation already starts early during CIOA (day 7, Figure 1A), while the onset of cartilage damage occurs around day 14. Moreover, synovial activation occurs early during CIOA and is reduced in S100A9 -/- mice (15). As such, we cannot completely rule out that S100A8/A9 could have its effect on osteophyte formation indirectly via synovial activation. Osteophyte size was not reduced in S100A9 -/- mice during DMM OA. Considering the near absence of synovitis during DMM, this again points to an important role for synovial activation in osteophyte formation. Although we have shown low S100A8/A9 levels in the synovium during DMM (15) a role for S100A8/A9 inside the chondrocyte on osteophyte formation cannot be fully excluded. Zreiqat et al. showed expression of S100A8 and S100A9 in chondrocytes during DMM (28). However, we were unable to detect S100A8/A9 in supernatants of human OA and murine chondrocytes nor show S100A8 or S100A9 on Western Blot in chondrocyte cell-lysates (unpublished results), indicating a marginal role for these alarmins in chondrocytes.

These results together suggest that synovial activation and consequently factors released by macrophages/infiltrated monocytes, such as S100A8 and S100A9, could stimulate osteophyte formation. Intra-articular injection and adenoviral

overexpression of both TGFβ1 and BMP-2 induces osteophyte formation in the mouse (17, 18, 29). In the current study, we could not find changes on synovial mRNA expression of TGFβ1 and BMP-2 nor levels of active TGFβ in S100A9 -/- during CIOA. TGFβ-levels however, are already high during CIOA and S100A8/A9 could be working as an enhancer to increase osteophyte size through catabolic actions.

Our lab has extensively shown that S100A8 and S100A9 are capable of upregulating and activating MMPs in chondrocytes (14, 30). From developmental knock-out studies in mice it is clear that MMPs are essential for proper endochondral ossification, a process similar to osteophyte formation (31, 32) and MMP-3 is strongly expressed within adult human osteophytic bone (33). In line with this, we showed in the present study that S100A8 could upregulate MMP-3 mRNA and activate MMPs in differentiating chondrocytes derived from murine MSCs. Moreover, we showed that VDIPEN expression and thus MMP activation is elevated in vivo during CIOA in the same areas in which chondrogenesis occurs. This supports the view that S100A8 and S100A9 can increase osteophyte size through activation of MMPs. By breaking down the cartilage matrix, MMPs could make it possible for chondrocytes to proliferate further and form larger osteophytes. These results together indicate that (MMP-mediated) catabolic rather than anabolic mechanisms are responsible for the proposed effects of S100A8/A9 on osteophyte formation.

CHECK has as main goal to identify factors important for the (early) onset and development of OA. Studies using the CHECK cohort of early symptomatic OA participants already identified several biomarkers and diagnostic tools for early OA (34, 35). Especially early diagnosis could vastly improve OA therapeutics, since it has proven almost impossible to reverse structural deformities in the later stages of the disease and clinicians often revert to total joint replacement. We found that plasma S100A8/A9 is significantly higher in CHECK participants that develop osteophytes, but remarkably this is not the case for CRP, ESR and COMP. Plasma S100A8/A9 higher than 200ng/ml also indicate a 4 times higher risk of developing osteophytes at later stages. Recently, we also showed a thickened intima lining of the synovium of CHECK participants that correlated with S100A8/A9 expression on immunohistochemistry. Also, we showed that high serum S100A8/A9 levels correlate with the development of cartilage damage in symptomatic OA patients (15). This study further increases the value

of measuring S100A8/A9 at an early stage of OA as possible predictor of both cartilage damage and osteophyte formation, although the number of participants (n=58) needs to increase to be able to assess proper prognostic value of S100A8/A9.

Taken together the current study shows that S100A8 and S100A9, as important products of activated macrophages during synovial activation in OA, could increase osteophyte size during experimental OA with synovial inflammation. S100A8/A9 can upregulate and activate MMPs that aid in the remodeling of the cartilage matrix, allowing for osteophytes to increase in size. Finally, we showed that S100A8/A9 may prove an interesting biomarker to not only predict cartilage damage, but also osteophyte progression during human OA.

Acknowledgements

The authors would like to thank Terry Jorna, Laurie de Kroon, Birgitte Walgreen and Monique Helsen for their excellent technical assistance and Janet Wesseling for cooperation in the CHECK cohort.

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Supplemental data

Supplemental Methods

RT-qPCR primer sequences

Primer sequences for target genes for RT-qPCR spanned exon-exon transitions and were as follows: TGFβ1; FW: GCAGTGGCTGAACCAAGGA, RV: AAGAGCAGTGAGCGCTGAATC. BMP-2; FW: CGCAGCTTCCATCACGAA, RV: GCCGGGCCGTTTTCC. BMP-6; FW: GATGGCAGGACTGGATCATTG, RV: AGGAACACTCTCCATCACAGTAGTTG. MMP-2; FW: CATGCGGAAGCCAAGATGT, RV: AGGTGTGTAACCAATGATCCTGTATGT. MMP-3; FW: TGAAGCCACCAACATCAGGA, RV: TGGAGCTGATGCATAAGCCC. MMP-9; FW: GGAACTCACACGACATCTTCCA, RV: GAAACTCACACGCCAGAAGAATTT. MMP-13; FW: AGACCTTGTGTTTGCAGAGCACTAC, RV: CTTCAGGATTCCCGCAAGAG.

Measurement of TGFβ activity

TGFβ activity was determined by adding 1:5 dilution of synovial washouts of CIOA mice (36) overnight to 3T3 fibroblasts transduced with adenoviral CAGA-luciferase (CAGA-luc) with a multiplicity of infection (MOI) of 10 (10 plaque-forming units) per cell, after which luminescence was measured. The plasmid was kindly provided by Dr. Ten Dijke (Department of Molecular Cell Biology, Leiden University Medical Center, The Netherlands). The CAGA-boxes in the vector are transcribed by Smad3/4 through active TGFβ, resulting in luciferase activity (37).

Measurement of S100A8/A9 protein

S100A8/A9 concentrations were determined in serum of mice with experimental OA or plasma of human CHECK samples and in synovial washouts of murine knee joints by a sandwich enzyme-linked immunosorbent assay (ELISA) specifically for either murine or human S100A8/A9 as described previously (38).

CHECK cohort

CHECK is a Dutch prospective cohort study of a total of 1,002 individuals with early symptomatic OA of the knee or hip (39). The CHECK cohort was initiated by the Dutch Arthritis Association and performed at Erasmus Medical Centre Rotterdam, Kennemer Gasthuis Haarlem, Leiden University Medical Centre, Maastricht University Medical Centre, Martini Hospital Groningen/Allied Health Care Centre for Rheumatology and Rehabilitation Groningen, Medical Spectrum Twente Enschede, Ziekenhuisgroep Twente Almelo, Reade/Jan van Breemen Research Institute and VU Medical Center Amsterdam, St. Maartenskliniek Nijmegen, University Medical Centre Utrecht, and Wilhelmina Hospital Assen.

Statistical analysis

Statistical differences were calculated using an unpaired t-test unless stated otherwise with Graph Pad Prism 5 (GraphPad Software). Differences were called significant with P-value < 0.05 (*), < 0.01 (**) or <0.0005(***).

Ethics

All animal experiments were approved by the local ethics committee of the Radboud University Nijmegen Medical Centre. The human study was approved by the medical ethics committees of all participating centers, and all participants gave their written informed consent before entering the study.

Supplemental Tables and Figures

Variable	Progressors	Non-progressors	Significance
Gender (% male vs female)	21% vs. 79%	21% vs. 79%	-
Age	57,4 (+/- 4,8)	56,1 (+/- 5,2)	ns
BMI	28,8 (+/- 4,9)	26,7 (+/- 3,3)	ns
Serum cholesterol (mmol/L)	56,8 (+/- 22,9)	53,2 (+/- 18,5)	ns
Total osteophyte BL	0,478 (+/- 0,39)	s0,198 (+/- 0,30)	*

Table S1: Demographic data participants CHECK cohort
Demographic parameters were not different between progressors (n=34) and non-progressors (n=24). BMI, Body Mass Index; BL, baseline. Total osteophyte score calculated by taking average osteophyte scores (0-3) of 4 locations per knee (medial and lateral tibia/femur) left and right. P-values were determined using Mann-Whitney test. ns=non-significant *=P<0.05

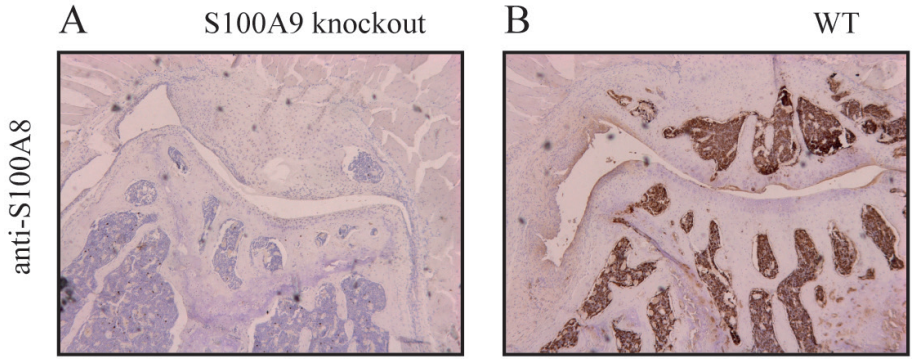


Figure S1: S100A8 protein is very sparsely expressed in the joint of S100A9 knockout mice.
Paraffin-embedded sections of S100A9 knockout (**A**) and WT mice (C57Bl/6J) on day 21 of collagenase-induced OA (**B**) were stained with specific anti-rabbit S100A8. **A**, S100A8 proteinwas sparsely expressed only in the bone marrow, not in other cells. **B**, S100A8 protein is abundantly expressed in synovial lining, bone marrow and growth-plate mostly.

References

1. de Lange-Brokaar BJE, Ioan-Facsinay A, van Osch GJVM, Zuurmond AM, Schoones J, Toes REM, et al. Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. Osteoarthritis Cartilage. 2012;20(12):1484-99.

2. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). Osteoarthritis Cartilage. 2013;21(1):16-21.

3. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. Bone. 2012;51(2):249-57.

4. Bondeson J, Blom AB, Wainwright S, Hughes C, Caterson B, van den Berg WB. The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis. Arthritis Rheum. 2010;62(3):647-57.

5. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis -- results of a 1 year longitudinal arthroscopic study in 422 patients. Osteoarthritis Cartilage. 2005;13(5):361-7.

6. Guermazi A, Roemer FW, Hayashi D, Crema MD, Niu J, Zhang Y, et al. Assessment of synovitis with contrast-enhanced MRI using a whole-joint semiquantitative scoring system in people with, or at high risk of, knee osteoarthritis: the MOST study. Ann Rheum Dis. 2011;70(5):805-11.

7. Conaghan PG, D'Agostino MA, Le Bars M, Baron G, Schmidely N, Wakefield R, et al. Clinical and ultrasonographic predictors of joint replacement for knee osteoarthritis: results from a large, 3-year, prospective EULAR study. Ann Rheum Dis. 2010;69(4):644-7.

8. Blom AB, van Lent PL, Libregts S, Holthuysen AE, van der Kraan PM, van Rooijen N, et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. Arthritis Rheum. 2007;56(1):147-57.

9. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol. 2009;86(3):557-66.

10. Foell D, Wittkowski H, Vogl T, Roth J. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. J Leukoc Biol. 2007;81(1):28-37.

11. Sunahori K, Yamamura M, Yamana J, Takasugi K, Kawashima M, Yamamoto H, et al. The S100A8/A9 heterodimer amplifies proinflammatory cytokine production by macrophages via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase in rheumatoid arthritis. Arthritis Res Ther. 2006;8(3):R69.

12. Hammer HB, Odegard S, Syversen SW, Landewe R, van der Heijde D, Uhlig T, et al. Calprotectin (a major S100 leucocyte protein) predicts 10-year radiographic progression in patients with rheumatoid arthritis. Ann Rheum Dis. 2010;69(1):150-4.

13. Brun JG, Jonsson R, Haga HJ. Measurement of plasma calprotectin as an indicator of arthritis and disease activity in patients with inflammatory rheumatic diseases. J Rheumatol. 1994;21(4):733-8.

14. Schelbergen RFP, Blom AB, van den Bosch MHJ, Sloetjes A, Abdollahi-Roodsaz S, Schreurs BW, et al. Alarmins S100A8 and S100A9 elicit a catabolic effect in human osteoarthritic chondrocytes that is dependent on Toll-like receptor 4. Arthritis Rheum. 2012;64(5):1477-87.

15. van Lent PLEM, Blom AB, Schelbergen RFP, Sloetjes A, Lafeber FPJG, Lems WF, et al. Active involvement of alarmins S100A8 and S100A9 in the regulation of synovial activation and joint destruction during mouse and human osteoarthritis. Arthritis Rheum. 2012;64(5):1466-76.

16. van der Kraan PM, van den Berg WB. Osteophytes: relevance and biology. Osteoarthritis Cartilage. 2007;15(3):237-44.

17. van Beuningen HM, Glansbeek HL, van der Kraan PM, van den Berg WB. Differential effects of local application of BMP-2 or TGF-beta 1 on both articular cartilage composition and osteophyte formation. Osteoarthritis Cartilage. 1998;6(5):306-17.

18. Blaney Davidson EN, Vitters EL, van Beuningen HM, van de Loo FAJ, van den Berg WB, van der Kraan PM. Resemblance of osteophytes in experimental osteoarthritis to transforming growth factor beta-induced osteophytes: limited role of bone morphogenetic protein in early osteoarthritic osteophyte formation. Arthritis Rheum. 2007;56(12):4065-73.

19. Blom AB, van Lent PLEM, Holthuysen AEM, van der Kraan PM, Roth J, van Rooijen N, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. Osteoarthritis Cartilage. 2004;12(8):627-35.

20. van der Kraan PM, Vitters EL, van Beuningen HM, van de Putte LB, van den Berg WB. Degenerative knee joint lesions in mice after a single intra-articular collagenase injection. A new model of osteoarthritis. J Exp Pathol (Oxford). 1990;71(1):19-31.

21. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. Osteoarthritis Cartilage. 2007;15(9):1061-9.

22. de Munter W, Blom AB, Helsen MM, Walgreen B, van der Kraan PM, Joosten LA, et al. Cholesterol accumulation caused by low density lipoprotein receptor deficiency or a cholesterol-rich diet results in ectopic bone formation during experimental osteoarthritis. Arthritis Res Ther. 2013;15(6):R178.

23. van Spil WE, Jansen NWD, Bijlsma JWJ, Reijman M, DeGroot J, Welsing PMJ, et al. Clusters within a wide spectrum of biochemical markers for osteoarthritis: data from CHECK, a large cohort of individuals with very early symptomatic osteoarthritis. Osteoarthritis Cartilage. 2012;20(7):745-54.

24. Altman RD, Gold GE. Atlas of individual radiographic features in osteoarthritis, revised. Osteoarthritis Cartilage. 2007;15 Suppl A:A1-56.

25. Manitz M-P, Horst B, Seeliger S, Strey A, Skryabin BV, Gunzer M, et al. Loss of S100A9 (MRP14) results in reduced interleukin-8-induced CD11b surface expression, a polarized microfilament system, and diminished responsiveness to chemoattractants in vitro. Mol Cell Biol. 2003;23(3):1034-43.

26. Blaney Davidson EN, Vitters EL, van der Kraan PM, van den Berg WB. Expression of transforming growth factor-beta (TGFbeta) and the TGFbeta signalling molecule SMAD-2P in spontaneous and instability-induced osteoarthritis: role in cartilage degradation, chondrogenesis and osteophyte formation. Ann Rheum Dis. 2006;65(11):1414-21.

27. Mort JS, Magny MC, Lee ER. Cathepsin B: an alternative protease for the generation of an aggrecan 'metalloproteinase' cleavage neoepitope. Biochem J. 1998;335 (Pt 3):491-4.

28. Zreiqat H, Belluoccio D, Smith MM, Wilson R, Rowley LA, Jones K, et al. S100A8 and S100A9 in experimental osteoarthritis. Arthritis Res Ther. 2010;12(1):R16.

29. Lories RJU, Luyten FP. Bone morphogenetic proteins in destructive and remodeling arthritis. Arthritis Res Ther. 2007;9(2):207.

30. van Lent PLEM, Grevers LC, Blom AB, Arntz OJ, van de Loo FAJ, van der Kraan P, et al. Stimulation of chondrocyte-mediated cartilage destruction by S100A8 in experimental murine arthritis. Arthritis Rheum. 2008;58(12):3776-87.

31. Krane SM, Inada M. Matrix metalloproteinases and bone. Bone. 2008;43(1):7-18.

32. Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, et al. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. Development. 2004;131(23):5883-95.

33. Bord S, Horner A, Hembry RM, Compston JE. Stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) expression in developing human bone: potential roles in skeletal development. Bone. 1998;23(1):7-12.

34. Vos PAJM, Welsing PMJ, deGroot J, Huisman AM, Oostveen JCM, Reijman M, et al. Skin pentosidine in very early hip/knee osteoarthritis (CHECK) is not a strong independent predictor of radiographic progression over 5 years follow-up. Osteoarthritis Cartilage. 2013;21(6):823-30.

35. Kinds MB, Marijnissen ACA, Vincken KL, Viergever MA, Drossaers-Bakker KW, Bijlsma JWJ, et al. Evaluation of separate quantitative radiographic features adds to the prediction of incident radiographic osteoarthritis in individuals with recent onset of knee pain: 5-year follow-up in the CHECK cohort. Osteoarthritis Cartilage. 2012;20(6):548-56.

36. Van Meurs JB, Van Lent PL, Joosten LA, Van der Kraan PM, Van den Berg WB. Quantification of mRNA levels in joint capsule and articular cartilage of the murine knee joint by RT-PCR: kinetics of stromelysin and IL-1 mRNA levels during arthritis. Rheumatol Int. 1997;16(5):197-205.

37. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. Embo J. 1998;17(11):3091-100.

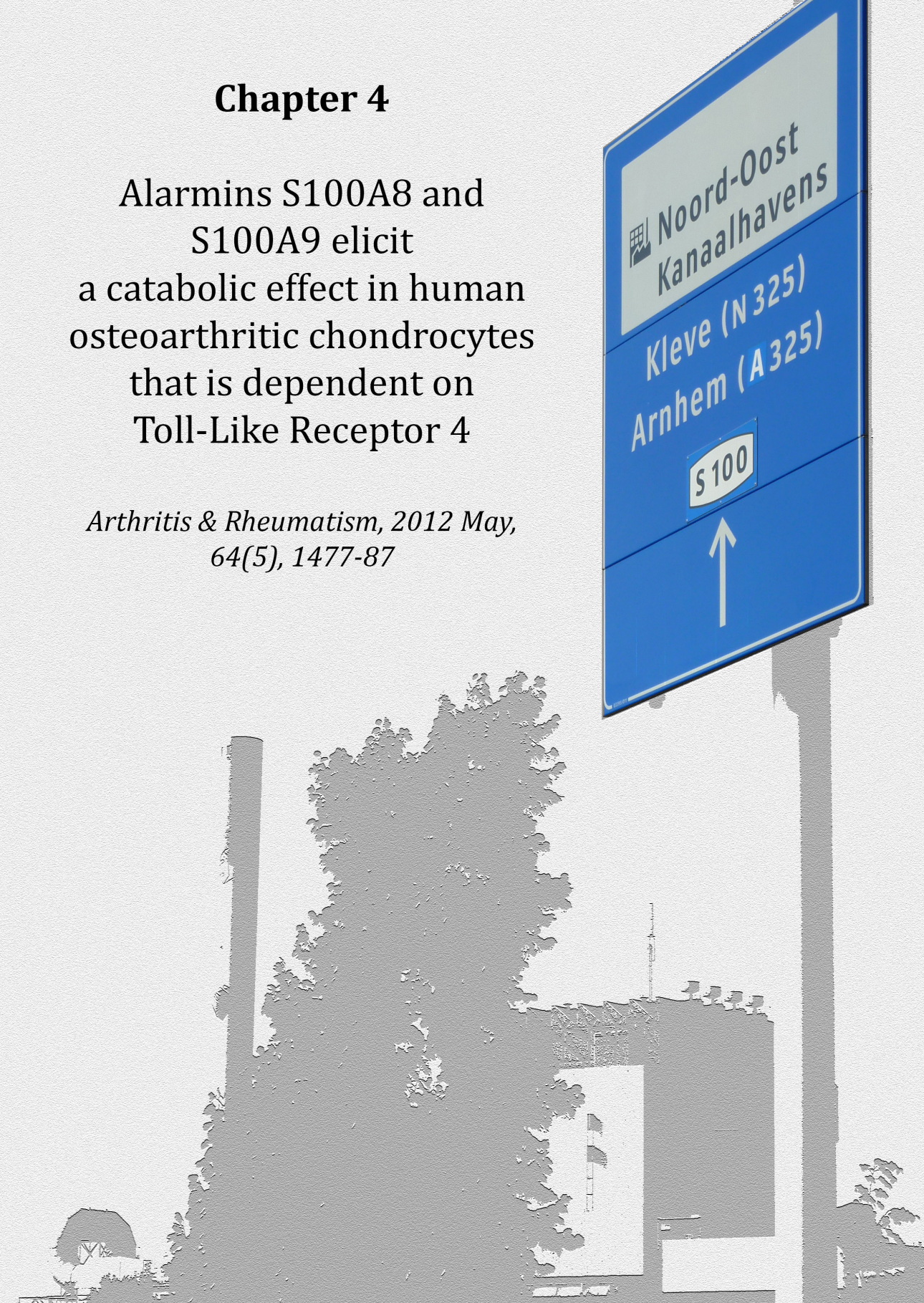
38. Wulffraat NM, Haas PJ, Frosch M, De Kleer IM, Vogl T, Brinkman DMC, et al. Myeloid related protein 8 and 14 secretion reflects phagocyte activation and correlates with disease activity in juvenile idiopathic arthritis treated with autologous stem cell transplantation. Ann Rheum Dis. 2003;62(3):236-41.

39. Wesseling J, Dekker J, van den Berg WB, Bierma-Zeinstra SMA, Boers M, Cats HA, et al. CHECK (Cohort Hip and Cohort Knee): similarities and differences with the Osteoarthritis Initiative. Ann Rheum Dis. 2009;68(9):1413-9.

Chapter 4

Alarmins S100A8 and S100A9 elicit a catabolic effect in human osteoarthritic chondrocytes that is dependent on Toll-Like Receptor 4

Arthritis & Rheumatism, 2012 May, 64(5), 1477-87



Chapter 4

Alarmins S100A8 and S100A9 elicit a catabolic effect in human osteoarthritic chondrocytes that is dependent on Toll-Like Receptor 4

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Abstract

Objective: S100A8 and S100A9 are two Ca^{2+}-binding proteins classified as damage-associated molecular patterns or alarmins that are found in high amounts in the synovial fluid of osteoarthritis (OA) patients. The purpose of this study was to investigate whether S100A8 and/or S100A9 can interact with chondrocytes from OA patients to increase catabolic mediators.

Methods: Using immunohistochemistry, we stained for S100A8 and S100A9 protein, matrix metalloproteinases (MMPs), and a cartilage-breakdown epitope specific for MMPs (VDIPEN) in cartilage from OA donors. Isolated chondrocytes or explants from OA and non-OA donors were stimulated with S100A8 and/or S100A9. Messenger RNA and protein levels of MMPs, cytokines, and cartilage matrix molecules were determined with quantitative reverse transcription–polymerase chain reaction and Luminex techniques, respectively. For receptor blocking studies, specific inhibitors for Toll-like receptor 4 (TLR-4), receptor for advanced glycation end products (RAGE), and carboxylated glycans were used.

Results: In cartilage from OA patients, the expression of S100A8 and S100A9 protein close to chondrocytes was associated with proteoglycan depletion and expression of MMP-1, MMP-3, and VDIPEN. Stimulation of chondrocytes with S100A8 and S100A9 caused a strong up-regulation of catabolic markers (MMPs 1, 3, 9, and 13, interleukin-6 (IL-6), IL-8, and monocytes chemotactic protein 1) and down-regulation of anabolic markers (aggrecan and type II collagen), thereby favoring cartilage breakdown. Blocking TLR-4, but not carboxylated glycans or RAGE, inhibited the S100 effect. The catabolic S100 effect was significantly more pronounced in chondrocytes from OA patients as compared to those from non-OA patients, possibly due to higher TLR-4 expression.

Conclusion: S100A8 and S100A9 have a catabolic effect on human chondrocytes that is TLR-4 dependent. OA chondrocytes are more sensitive than normal chondrocytes to S100 stimulation.

Introduction

During osteoarthritis (OA) the erosion of cartilage is an important cause of pain and disability in the joint. Cartilage damage associated with OA is mainly caused by a shift in the balance between resorptive (or catabolic) and synthetic (or anabolic) capacities of chondrocytes. Catabolic activities of OA chondrocytes are characterized by elevated release of cartilage degrading enzymes, such as matrix metalloproteinases (MMPs), while anabolic activity of chondrocytes results in aggrecan and collagen type II production (1). Although OA is generally considered as a disease of the cartilage, recent evidence is accumulating that synovial activation and pro-inflammatory mediators released after synovial activation might contribute to induction of cartilage damage in OA (2-4). Arthroscopic studies suggest that synovial activation occurs in 50% of OA patients and that it is associated with more severe cartilage damage (5). In line with this, pro-inflammatory mediators released by synovial macrophages or fibroblasts contribute to cartilage damage, although the involvement of for example interleukin-1 (IL-1), tumor necrosis factor α (TNF-α) and IL-6 is still a matter of debate (6;7).

Apart from cytokines, damage associated molecular patterns (DAMPs), also termed alarmins, are released after activation of the synovium. Major members of the DAMP-family are the calcium binding proteins S100A8 (also: Myeloid Related Protein (MRP) 8) and its binding partner S100A9 (also: MRP14) (8-10). They belong to the S100-family comprising at least 20 proteins (11) and are released in large amounts by neutrophils, monocytes and activated macrophages (12). S100A8 and S100A9 exist as homodimers and can form S100A8/S100A9 heterodimer complexes. They can bind to Toll-like receptor 4 (TLR4) (13), but associations with the receptor for advanced glycation end products (RAGE) and carboxylated glycans have also been described (14;15).

In rheumatoid arthritis (RA), the S100A8/A9 heterodimer accumulates in huge amounts (up to 50 μg/ml) in synovial fluid and its levels strongly correlate with disease activity and radiological damage (8;16). Studies on serum levels of the S100A8/S100A9 heterodimer in RA confirm this correlation and highly support the diagnostic capacity of S100A8/S100A9 as a biomarker in RA (17;18). More

recently, we demonstrated that in arthritic mice deficient for S100A9 (in which myeloid cells lack S100A8 protein) cartilage destruction was almost absent and joint inflammation significantly reduced (19).

The role of S100A8 and S100A9 in OA is still enigmatic and evidence of its involvement mainly comes from the effects of S100A8 and S100A9 on chondrocytes in vitro. On murine and ovine chondrocytes S100A8 and S100A9 upregulate several MMPs (14;20). And one study shows the presence of S100A8 and S100A9 in murine chondrocytes during destabilization of the medial meniscus (DMM) OA (20).

In the present study, we investigated whether S100A8 and/or S100A9 is present in human OA cartilage and whether it is associated with cartilage breakdown. Moreover, we used human chondrocytes to explore the in vitro capacity of S100A8, S100A9 and/or the S100A8/S100A9 complex to induce a more catabolic chondrocyte. Finally, we investigated possible receptors and whether there is a difference in sensitivity between OA and normal chondrocytes. In short, our results indicate that S100A8 and S100A9 are associated with cartilage breakdown in human OA tissue and that they stimulate chondrocytes from OA patients to produce more catabolic factors (MMPs and cytokines) and less anabolic factors (collagen type II and aggrecan). Furthermore, the net catabolic effect was dependent on TLR4 and significantly higher in OA chondrocytes compared to normal chondrocytes.

Materials and Methods

S100A8/A9 proteins

Recombinant human S100A8, S100A9 and S100A8/A9 heterodimer complex with no LPS contamination were expressed and purified as previously described (21;22). Protein preparations were tested using the Limulus amoebocyte cell lysate (BioWhittaker, Walkersville, MD), and lipopolysaccharide (LPS) content was undetectable (sensitivity 0.7 ± 0.5 pg LPS/μg protein), indicating that the maximal possible contamination of the S100 protein preparations was <1.2 pg LPS/μg protein. S100A8 and S100A9 lost their activity while heat-inactivating

at 80°C for 30 minutes. LPS activity was not changed at that temperature (13).

Tissue acquisition and chondrocyte cell culture

Human cartilage was collected from patients with OA undergoing total knee or hip arthroplasty or from age-matched non-OA patients suffering a femoral neck fracture of the hip. After collection, either cartilage explants of approximately 2 x 2 mm were cut and cultured in Dulbecco's modified Eagle's medium (DMEM) F12 (Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS), or chondrocytes were isolated. For isolation, cartilage was incubated overnight with 2 mg/ml collagenase B (Roche, Mannheim, Germany) in serum-free DMEM F12 medium. The next day, the cells were washed and seeded on 24-well plates at 4x105 cells/well in DMEM F12 medium supplemented with 10% FCS. Plates were maintained for 5-7 days prior to stimulations. The Medical Ethics Committee of Radboud University Nijmegen Medical Centre approved the study protocol.

Stimulation of chondrocytes and cartilage explants

Monolayer chondrocyte cultures were stimulated with recombinant human S100A8, S100A9 and the S100A8/A9 complex at 1 μg/ml (unless stated otherwise) and 10 ng/ml human recombinant IL-1β (R&D Systems, Minneapolis, MN) for 24 hours. Optimal concentrations for S100 stimulations were determined by stimulating OA chondrocytes with 0.2, 1 and 5 μg/ml S100A8 and S100A9 and measuring IL-6, MMP3 and -13 mRNA levels with quantitative reverse transcriptase PCR (RT-qPCR). In the blocking studies against TLR4, RAGE and carboxylated glycans, chondrocytes were pre-incubated for 30 minutes with specific blockers before stimulation with S100-proteins or IL-1β. For TLR4, 1 μM of the small molecule inhibitor TAK242 was used (23) (CLI-095, InvivoGen, San Diego, CA), for RAGE a blocking anti-human RAGE antibody, 10 μg/ml (R&D Systems (MAB11451), Minneapolis, MN) and for carboxylated glycans 10 μg/ml of the monoclonal antibody GB3.1 was used (24) (kind gift of G. Srikrishna and H. Freeze, Sanford Children's Health Research Center, La Jolla, CA). In all stimulations with S100A8 and/or S100A9 and IL-1β, 10 μg/ml polymixin B was added (Sigma, Deisenhofen, Germany).Cartilage explants were maintained in culture for 1 day after collection, then stimulated for 24 hours with the same concentrations of S100 proteins and IL-1β as described above.

Quantitative detection of messenger RNA (mRNA) levels using RT-qPCR

RNA was isolated from chondrocytes with 1 ml of TRI-reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands). Specific mRNA levels for cytokines and chemokines (IL-6, IL-8, MCP-1, IL-1β, TNFα), MMPs (MMP-1, MMP-3, MMP-9, MMP-13), aggrecan and collagen type II were detected using the ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Briefly, 1 μg of total RNA was used for RT-PCR. mRNA was reverse transcribed to complementary DNA (cDNA) using oligo(dT) primers, and 1/20 of the cDNA was used in 1 PCR reaction. PCR was performed in SYBR Green Master Mix using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with data collection in the last 30 seconds. mRNA for human genes was amplified using specific primers (Biolegio, Malden, The Netherlands) at a final concentration of 300 nmoles/liter. Relative quantification of the PCR signals was calculated by comparing the threshold cycle (Ct) value of the different genes, correcting for GAPDH content (dCt) and finally correcting for unstimulated conditions (ddCt).

Protein measurement using Luminex technology

To determine protein levels of MMPs (MMP-1, MMP-3, MMP-9, MMP-13) and cytokines/chemokines (IL-6, IL-8 and MCP-1) in chondrocyte culture medium, Luminex multianalyte technology on the Bio-Plex system was used in combination with multiplex MMP/cytokine kits (Milliplex from Millipore, Amsterdam, The Netherlands). Protein levels were measured in 25 μl of culture medium, diluted 1:5. The sensitivity of the multiplex kit was <5 pg/ml.

Immunohistochemical detection of VDIPEN, S100 and MMPs in paraffin sections

Formalin-fixed specimen of cartilage from either OA or non-OA were paraffin embedded and 7 μm thick sections were cut. Serial sections were digested with proteinase-free chondroitinase ABC (0.25 units/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) to remove the glycosaminoglycan side chains. For VDIPEN, cartilage sections were incubated with rabbit anti-VDIPEN, for S100A8 and S100A9 goat polyclonal antibody anti-human S100A8 and S100A9, for MMP3 goat polyclonal anti-human MMP3 (all Santa Cruz Biotechnology,

Santa Cruz, CA) and for MMP1 goat polyclonal anti-human MMP1 (R&D Systems, Minneapolis, MN). The primary antibody was detected using biotinylated rabbit anti-goat, except for VDIPEN for which we used biotinylated goat anti-rabbit IgG and subsequently stained using avidin–streptavidin–peroxidase (Elite kit; Vector, Burlingame, CA). Orange G (2%) was used for counterstaining the VDIPEN sections, Mayer's hematoxylin (Merck, Darmstadt, Germany) for S100A8 and S100A9 and Toluidine Blue for MMP1 and MMP3.

Statistical analysis

Statistical differences were calculated with either paired t-test or Mann Whitney U test, as indicated, using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA). Differences were called significant with P-values < 0.05.

Results

Localization of S100A8 and S100A9 proteins in human OA cartilage and association with proteoglycan depletion and MMP expression

Alarmins S100A8 and S100A9 are found in high concentration (up to 5 μg/ml) in the synovial fluid of OA patients, possibly reflecting synovial activation (8). We analyzed whether S100A8 and S100A9 are found in human OA cartilage. For this, cartilage specimens obtained following hip or knee arthroplasty of several OA patients (n=8) were embedded in paraffin and sliced into 7 μm thick serial sections. When stained with antibodies against S100A8 and S100A9, we found that S100A8 is abundantly present close to the majority of the chondrocytes (>75%) in the superficial layer of the cartilage (Figure 1A). Identical staining was found for S100A9 (data not shown). When comparing S100 localization with safranin O staining (Figure 1B) we observed that S100A8 and S100A9 were only found in areas with PG loss. We also found staining of the MMP-induced neo-epitope VDIPEN in the same areas as S100A8 and S100A9 expression (Figure 1C). Finally, MMP1 and MMP3 were also detected in the majority of the chondrocytes (>75%) in the superficial layer (Figure 1D-E), their expression pattern coinciding with that of S100A8 and S100A9. These results suggest that S100A8 and S100A9 proteins are expressed in similar areas of OA cartilage in which there is PG loss

and MMP mediated cartilage destruction.

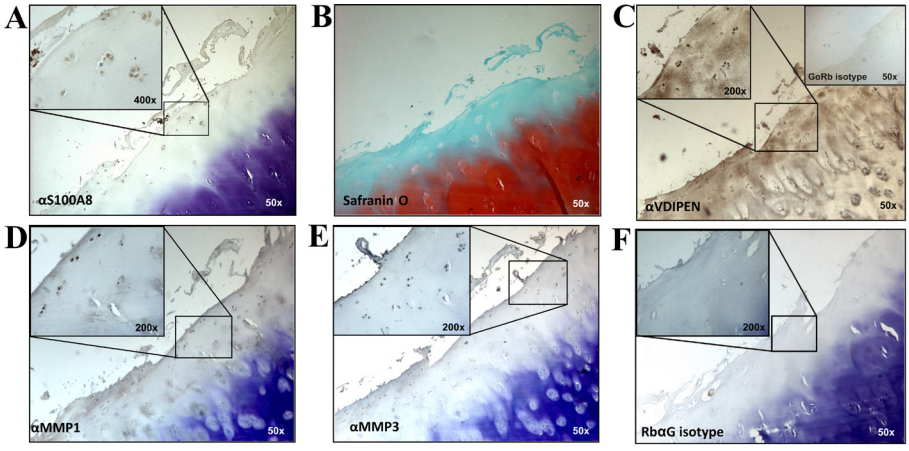


Figure 1 – Expression of S100A8 and several cartilage breakdown markers in adjoining sections of human articular cartilage from OA patients as measured by immunolocalisation.
OA cartilage was embedded in paraffin and sliced into 7µm thick serial sections. S100A8 is clearly seen close to the majority of (>75%) of the chondrocytes in the superficial layer of the cartilage (A). Expression of S100A9 shows the same pattern (not shown). Safranin-O staining of proteoglycans is observed in the deep layers (B). There is no proteoglycan staining in the superficial layer where we found S100A8 and S100A9 expression in A. VDIPEN staining as measure of MMP activation is also found in the same areas as S100A8 and S100A9 in A (C). Isotype staining (top-right inset) is negative. MMP1 and MMP3 is also seen in the majority of (>75%) of the chondrocytes in the superficial layer of the cartilage (D-E). Isotype staining for MMP1, MMP3 and S100A8 is negative (F). Magnifications are depicted in the bottom right corner. Staining patterns are representative for 8 OA donors.

S100A8 and S100A9 stimulate breakdown of human cartilage matrix in situ through matrix metalloproteinases

Next, we wondered whether chondrocytes could be stimulated by S100 proteins in intact cartilage. Small proteins like S100A8 and S100A9 (homodimeric size 24 kD) have been shown to easily penetrate intact cartilage and stimulate chondrocytes (25). When we stimulated cartilage explants with 1 µg/ml S100A8 and S100A9, mRNA levels of MMP1, -9 and -13 and IL-6 were significantly upregulated compared to non-stimulated controls (Figure 2A). Moreover, on mRNA level S100A8 significantly downregulated aggrecan and collagen type II (Figure 2B).We concluded that S100A8 and S100A9 can have a catabolic effect on chondrocytes in situ in OA cartilage.

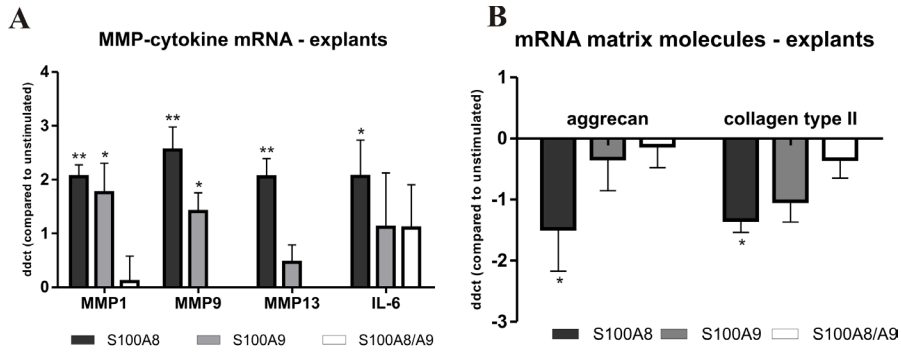


Figure 2 – S100A8 and S100A9 have a catabolic effect on human cartilage explants as seen by upregulation of MMP-1, -9, -13 and IL-6 mRNA expression and downregulation of aggrecan and collagen type II mRNA expression.
Cartilage explants (approximately 2x2 mm) were excised from cartilage derived from patients undergoing total hip arthroplasty following a femoral neck fracture (n=6). Explants were kept in culture for 24 hours before 24 hour stimulation with 1 μg/ml human recombinant S100A8, S100A9 or S100A8/A9 heterodimer and subsequent measurement of mRNA expression by qPCR. Catabolic enzymes MMP1, -9 and -13 and pro-inflammatory cytokine IL-6 are upregulated by S100A8 and S100A9, but not the S100A8/S100A9 heterodimer (**A**). Cartilage matrix components aggrecan and collagen type II are downregulated by S100A8 (**B**).* = $P < 0.05$ ** = $P < 0.01$ *** = $P < 0.005$ stimulated versus non-stimulated explants by paired t-test.

S100A8 and S100A9 suppression of the anabolic function of human OA chondrocytes

To investigate the effects of S100A8 and S100A9 on human chondrocytes in more detail, we isolated chondrocytes from OA patients (n=8), cultured them for 5-7 days and stimulated them for 24 hours with 1 μg/ml S100A8, S100A9 and the S100A8/A9 complex. This concentration is also physiologically relevant, since S100A8/S100A9 levels in OA synovial fluid range from a few to 10 μg/ml (8).We first studied the effect of the S100-proteins on the anabolic function of the chondrocyte. S100A8 and S100A9 significantly inhibited both aggrecan and collagen type II mRNA (S100A8 1.6 and 1.2 ddCt; S100A9 1.4 and 1.6 ddCt respectively) (Figure 3A). The inhibiting effect of S100A8 and S100A9 was compared with that of IL-1β, the most potent inhibitor for aggrecan and collagen type II synthesis. Although less potent than IL-1β, S100A8 and S100A9 effects were still 70 and 61% of the IL-1β effect for aggrecan and 26 and 35% for collagen type II.Our results indicate that S100A8 and S100A9 have a substantial suppressive effect on cartilage matrix synthesis by chondrocytes from OA patients.

Up-regulation of MMPs 1, 3, 9, and 13 following stimulation of human OA chondrocytes with S100A8 and S100A9

In addition, we studied the effect of S100A8, S100A9 and its heterodimeric complex on the catabolic functions of the chondrocyte, which are largely mediated by MMPs. We focused on MMP-1, -3, -9 and -13 since they are all upregulated during OA (26). In previous studies we have shown that murine S100A8 but not S100A9 strongly upregulate MMPs in a murine chondrocyte cell line (14).S100A8 and S100A9 significantly upregulated MMP1, MMP3, MMP9 and MMP13 at the mRNA level. For S100A8 2.0, 2.0, 1.5 and 1.0 ddCt respectively, for S100A9 even more: 3.0, 2.6, 2.5 and 2.2 ddCt respectively (Figure 3B). The upregulation of MMPs was between 12 and 30% of the effect of IL-1β (a strong inducer of MMPs) for S100A8 and 24 to 62% for S100A9.Whether MMP proteins were released by chondrocytes after stimulation with S100A8 and S100A9 was further investigated using a Luminex-based protein assay. MMP1, -3 and -13 protein levels in the culture medium were significantly elevated by S100A8 (2.1, 3.0 and 3.3 fold respectively) and even more by S100A9 (2.6, 3.1 and 3.9 fold respectively) (Figure 3C and 3D). Compared to IL-1β, the effect of S100A8 on MMP1, -3 and -13 protein level was 55, 28 and 30% respectively and 63, 29 and 26% for S100A9.
In conclusion, S100A8 and S100A9 stimulation of OA chondrocytes upregulates MMP expression on mRNA and protein level.

Up-regulation of IL-1β, IL-6, IL-8, and MCP-1 following stimulation of human OA chondrocytes with S100A8 and S100A9

Apart from MMPs, catabolic effects of chondrocytes are mediated through several cytokines and chemokines. At the mRNA level, S100A8 and S100A9 upregulated IL-1β and IL-6 but not TNFα (S100A8: 4.3, 3.4 and 0.4 ddCt; S100A9: 5.2, 4.4 and 0.5 ddCt respectively) (Figure 4A). Measurement of the corresponding protein levels in the culture supernatant showed that S100A8 and S100A9 strongly upregulated secretion of IL-6 by chondrocytes (23.7 and 34.4 fold respectively) (Figure 4B). However, IL-1β and TNFα protein levels were below the detection limit (data not shown). The chemokine IL-8, which earlier has been shown to induce hypertrophy in human articular chondrocytes (27), was significantly

upregulated upon stimulation with S100A8 and S100A9. At the mRNA level, IL-8 was upregulated by S100A8 (3.9 ddCt) and S100A9 (5.3 ddCt) (Figure 4C). In line with mRNA data, secreted IL-8 protein level was also elevated (20.4 and 79.0 fold for S100A8 and S100A9 respectively) (Figure 4D). MCP-1, a chemokine involved in migration of macrophages, was significantly upregulated at the mRNA level by S100A8 and S100A9 (1.7 and 2.3 cycles respectively) (Figure 4C), as well as at the protein level in the culture supernatant (6.7 and 8.7 fold respectively) (Figure 4D).

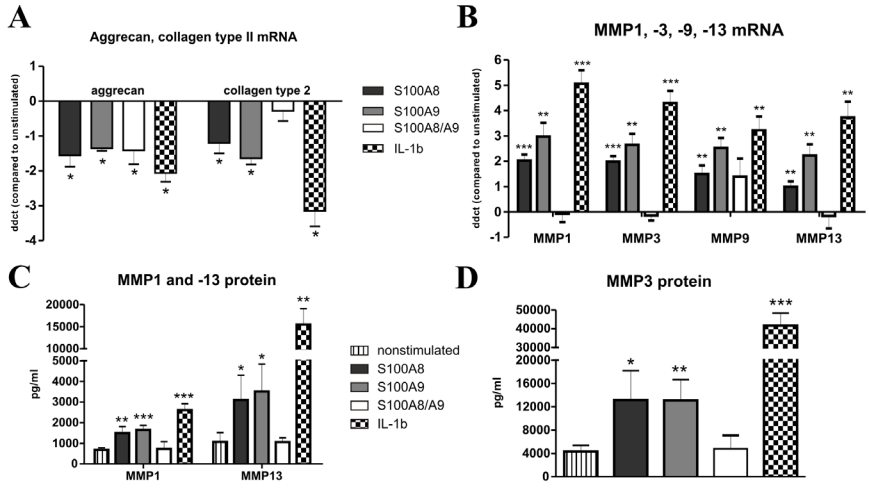


Figure 3 – S100A8 and S100A9 suppress mRNA levels of aggrecan and collagen type II and stimulate MMPs on mRNA level and on protein level in chondrocytes from OA patients.
After isolation from articular OA cartilage (n=8), chondrocytes were stimulated for 24 hours with 1 μg/ml S100A8, S100A9 or S100A8/A9 complex. 10 ng/ml IL-1β served as positive control. mRNA levels were measured with qPCR (**A, B**) and MMP1, -3 and -13 total protein level was determined in culture supernatants with a Luminex-based protein immunoassay (**C, D**). * = P < 0.05 ** = P < 0.01 *** = P < 0.005 stimulated versus non-stimulated (medium control) chondrocytes by paired t-test.

Taken together, our results indicate that S100A8 and S100A9 stimulate OA chondrocytes to produce increased levels of pro-inflammatory cytokines and chemokines. In sharp contrast to the strong stimulating effects of S100A8 and S100A9 homodimers, the heterodimeric S100A8/S100A9 complex did not show any significant effect on either MMPs (Figure 3) or cytokines and chemokines (Figure 4), neither on mRNA nor at the protein level.

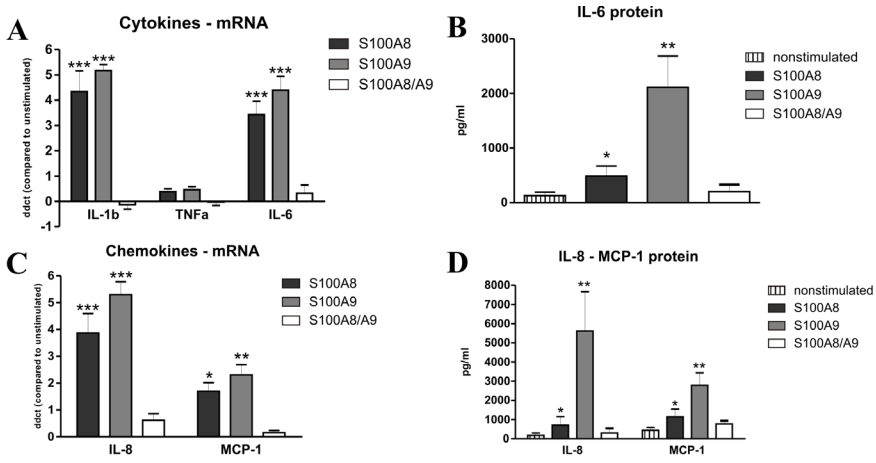


Figure 4 – S100A8 and S100A9 stimulate mRNA expression and protein release of several cytokines and chemokines in chondrocytes from OA patients.
After isolation from articular OA cartilage (n=8), chondrocytes were stimulated for 24 hours with 1 μg/ml S100A8, S100A9 or S100A8/A9 complex. mRNA levels of cytokines IL-1β, IL-6 and TNFα (**A**) and chemokines IL-8 and MCP-1 (**C**) were measured with qPCR. In the culture medium protein levels of IL-6 (**B**) and IL-8 and MCP-1 (**D**) were determined using a Luminex-based immunoassay. Protein levels of IL-1β and TNFα were undetectable in the culture medium. * = $P < 0.05$ ** = $P < 0.01$ *** = $P < 0.005$ stimulated versus non-stimulated chondrocytes by paired t-test.

Catabolic effect of S100A9 on OA chondrocytes via TLR-4

Various receptors like TLR4, RAGE and carboxylated glycans have been proposed to be involved in S100A8/A9 signaling. To investigate the involvement of these receptors, we stimulated human chondrocytes from several OA donors with S100A9 in the presence or absence of specific blockers. For TLR4 we used an intracellular small molecule inhibitor (TAK242), and for both RAGE and the carboxylated glycans a neutralizing antibody. We used S100A9 as stimulator, as it proved to be the most potent form in the previous experiments. Interestingly, blocking TLR4 with TAK242 almost completely suppressed S100A9 mediated elevation of MMP3 (79% inhibition), cytokine IL-6 (83% inhibition), chemokine MCP-1 (79% inhibition) and collagen type II (86% inhibition) (Figure 5A). The upregulation of MMP1, -9 and -13 by S100A9 was also blocked by adding TAK242, yet to a lesser extent (60%, 69%, 52%). However, the inhibition was comparable to that of the LPS effect (data not shown), suggesting that perhaps higher concentrations of TAK242 are necessary for complete inhibition. In

contrast, blocking of RAGE did not change S100A9-stimulated expression of MMPs, collagen type II or cytokines (Figure 5B), nor did blocking carboxylated glycans (Figure 5C). These data suggest that TLR4 is a candidate receptor for S100A8 and S100A9 signaling in human chondrocytes.

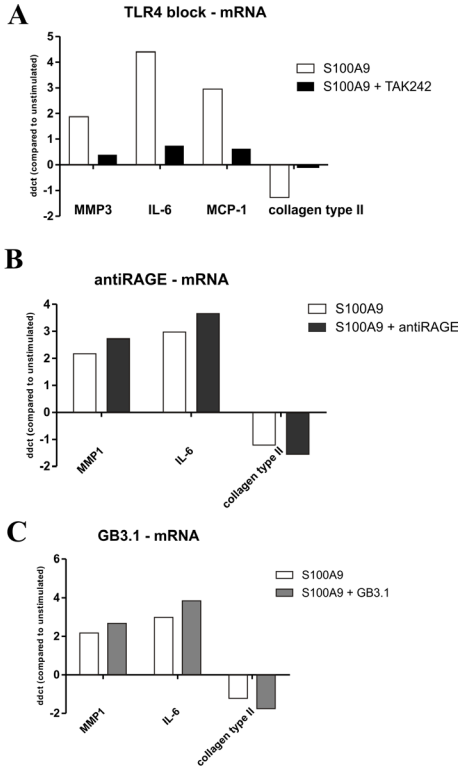


Figure 5 – The catabolic effect of S100A9 on OA chondrocytes is mediated through TLR4, not RAGE or carboxylated glycancs.
The effect of blocking TLR4 (**A**), RAGE (**B**) and carboxylated glycans (**C**) on S100A9 stimulation of OA chondrocytes (n=3). After isolation from articular cartilage, chondrocytes were stimulated for 24 hours with 1 μg/ml S100A9, with or without 30 minute pre-incubation with a specific blocker. (TAK242 for TLR4, anti-human RAGE blocking antibody for RAGE and GB3.1 antibody for carboxylated glycans). Transcript levels were measured with qPCR.

Significantly higher catabolic effect of S100A8 and S100A9 on OA chondrocytes than non-OA chondrocytes

To explore if chondrocytes from OA patients are more sensitive to S100A8 and S100A9, we compared the anabolic and catabolic effects of S100A8 and S100A9 on OA chondrocytes with those on non-OA chondrocytes. For this, we isolated chondrocytes from age-matched donors (n=4) without a history of OA that had suffered a femoral neck fracture. Interestingly, we found that inhibition of aggrecan mRNA by S100A8 and S100A9 was significantly higher in chondrocytes from OA patients compared to non-OA (1.4 and 1.0 ddCt difference respectively) (Figure 6A). S100A8 and S100A9 stimulation of non-OA chondrocytes induced

a minor increase in mRNA level of MMP1, -3, -9 and -13 and IL-6 (data not shown). When comparing the effects of S100A8 and S100A9 on OA and normal chondrocytes, MMP3 was upregulated significantly higher in OA chondrocytes (1.3 and 1.3 ddCt difference respectively) (Figure 6B). Although MMP1, -9 and -13 and IL-6 mRNA levels were somewhat higher in OA chondrocytes, this did not reach statistical significance. Since we showed that TLR4 is important for S100 signaling in human OA chondrocytes, we next wondered if differences in the level of TLR4 expression could account for the heightened sensitivity for S100 in OA chondrocytes. When comparing TLR4 mRNA levels in unstimulated chondrocytes between OA (n=8) and non-OA donors (n=4), we found that TLR4 mRNA is higher in OA chondrocytes, compared to non-OA (Figure 6C). These data suggest that TLR4 is upregulated on OA chondrocytes compared to non-OA chondrocytes possibly explaining why the catabolic effect of S100A8 and S100A9 is greater on OA chondrocytes than on non-OA chondrocytes.

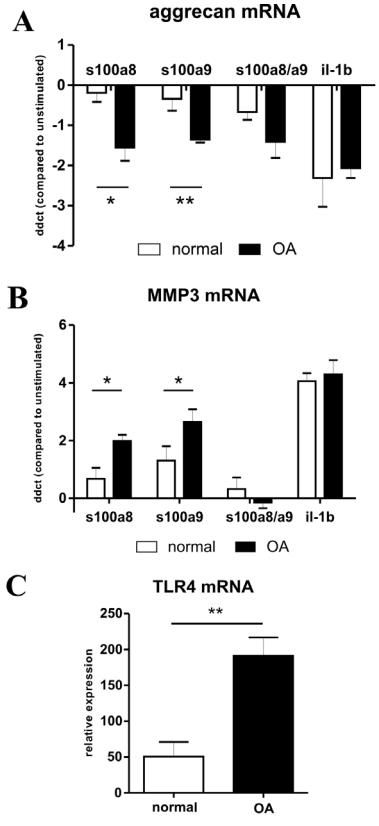


Figure 6 – The catabolic effect of S100A8 and S100A9 on human chondrocytes is significantly higher in OA-derived chondrocytes, compared to non-OA and possibly mediated through TLR4.
Cartilage from OA donors (n=8) and age-matched non-OA ("normal") donors (n=4) was collected after a hip-replacing operation following a femoral neck fracture. mRNA levels for the anabolic marker aggrecan (**A**) and the catabolic marker MMP3 (**B**) after S100A8, S100A9, S100A8/A9 stimulation were measured with qPCR. TLR4 mRNA levels were measured in unstimulated OA or non-OA chondrocytes. dCt values (corrected for GAPDH) are represented as relative expression ($=2^{-dCt}$) (**C**). * = P < 0.05 ** = P < 0.01 *** = P < 0.005 normal versus OA chondrocytes by Mann Whitney U test

Discussion

In the present study we show that S100A8 and S100A9 can elicit a TLR4-dependent catabolic effect in human chondrocytes which is significantly elevated under OA conditions.

S100A8 and S100A9 are mostly associated with inflammation as they are implicated in numerous inflammatory diseases (RA, systemic lupus erythematosus (SLE), and psoriasis (28;29)). In RA, S100A8 and S100A9 proteins are produced in large amounts by infiltrating myeloid cells and their concentration can reach up to 50 μg/ml in RA synovial fluid (8). Although it is generally accepted that OA is a disease of the cartilage, evidence is accumulating that synovitis also plays a significant role in OA (30). Histologically, OA patients of all severity grades show thickening of the synovial lining as well as increased inflammatory cell infiltration (31;32). The histological changes in OA are accompanied by an increase in inflammatory mediators in the synovium. OA synovium expresses significant amounts of the cytokines IL-1β, TNF and IL-6 as well as the chemokines IL-8 and MCP-1 (32-34). Among the most abundantly produced proteins in OA synovial fluid are the S100A8 and S100A9 proteins reaching concentrations up to 10 μg/ml (8). We now show that S100A8 and S100A9 strongly upregulate IL-6 , IL-8 and MCP-1 on mRNA and protein level in OA chondrocytes and IL-1β on mRNA level. Release of IL-8 by chondrocytes may attract granulocytes to the cartilage surface promoting cartilage destruction and increasing S100 production. IL-8 can further deteriorate cartilage integrity by causing chondrocyte hypertrophy through upregulation of collagen type X (35). MCP-1, which was also significantly upregulated by S100A8 and S100A9, is a major chemokine for monocytes/macrophages and may attract these S100A8/S100A9 producing cells towards the synovial compartment and cartilage surface. Together, the elevated production of IL-8 and MCP-1 might cause an increased inflammatory cell-influx and more S100A8/A9 production thereby possibly creating a positive feedback loop of inflammation and cartilage degradation.

Next to an effect on inflammation, we also found that S100A8 and S100A9 stimulate production of several MMPs (MMP1, -3, -9 and -13) by OA chondrocytes, both at the mRNA as well as the protein level. To our knowledge, this is the first

study in human chondrocytes that shows upregulation of MMPs by S100A8 and S100A9. Previous studies performed in the mouse show that S100A9 knock-out mice, whose myeloid cells are deficient for S100A8 protein, are protected against severe cartilage destruction during an antigen induced arthritis (19). Interestingly, S100A8 but not S100A9 stimulated MMP expression in an immortalized murine chondrocyte cell line (14). We now find, using human S100 proteins and human primary chondrocytes, that also S100A9 is a significant stimulator of MMP production. This is in line with earlier findings in primary ovine chondrocytes in which both S100A8 and S100A9 upregulated several MMPs at the mRNA level (20). The lack of effect of S100A9 in mouse could be due to the use of an immortalized murine chondrocyte line that expresses different receptors. Studies are now in progress in which primary murine chondrocytes are used for S100 stimulation. Apart from a stimulating effect on MMPs, we show that S100A8 and S100A9 downregulate the expression of the anabolic factors aggrecan and collagen type II in OA chondrocytes. This is also in line with earlier studies performed in ovine chondrocytes (20). Taken together, we demonstrated that S100A8 and S100A9 elicit a net catabolic response in OA chondrocytes.

In contrast to the homodimers S100A8 and S100A9, the S100A8/S100A9 heterodimer only had minor effects in the current study. The lack of activity of the S100A8/A9 complex may be dependent on the species and/or cell-type. In human endothelial cells, the S100A8/S100A9 heterodimer increased the inflammatory response (36). Sunahori et al. showed that the heterodimeric complex and the S100A9 homodimer were equally active on human monocytes and macrophages by increasing production of pro-inflammatory cytokines (8). A recent study by our lab showed that in the mouse only S100 homodimers stimulated the expression of activatory Fcγ receptors in macrophages (37). No effect of the S100A8/S100A9 heterodimer was found in murine nor ovine chondrocytes on pro-inflammatory cytokines and MMPs (14;20). This is in line with our results that show that the heterodimer is inactive on human primary chondrocytes. An explanation for the inactivity of the heterodimer might be that coupling of S100A8 to S100A9 shield the sites needed for receptor binding. Although not conclusively shown for S100A8 and S100A9 but known for other S100 proteins, the heterodimers may also require post-translational modification for proper functioning. S100A4 required sumoylation to upregulate MMP13 (38) and transamidation of S100A11 skewed chondrocytes towards a catabolic phenotype (39).

The receptor(s) responsible for S100A8 and S100A9 signaling in chondrocytes is still under debate. Up till now various receptors have been implicated in S100A8 and/or S100A9 signaling, e.g. TLR4, RAGE and carboxylated glycans. In the present study we show that blockade of TLR4, but not RAGE or carboxylated glycans, inhibited the effect of S100A9 on MMPs and IL-6 production, suggesting that in human chondrocytes TLR4 is a major receptor for S100A8 and S100A9 signaling. TLR4 is present in primary chondrocytes and is strongly stimulated by TLR ligands like LPS (40). TLR4 is the major receptor for S100A8 and S100A9 in other cell types, like macrophages. Vogl et al. showed that upregulation of NFκB by S100A8 is TLR4-dependent (13). Further study, performed in our lab, demonstrated that the upregulation of Fcγ receptors by S100A8 was completely absent in TLR4 knock-out macrophages (37). A few studies report the interaction of S100A8 and/or S100A9 with RAGE (41;42) but not in chondrocytes. Signaling through RAGE has only been showed for other S100 family members, most notably S100A11 and S100A4 (27;43). Combining these two observations, it is unlikely that RAGE plays a role in the signaling of S100A8 and S100A9 in chondrocytes. Carboxylated glycans mediate transendothelial leukocyte migration by S100A8/A9 (44). Contrasting the results shown in the current study, earlier studies in our lab show that carboxylated glycans are also important in S100A8 signaling in a murine chondrocyte cell-line (14). However, immortalization of this cell-line caused low expression levels of TLR4 and internalization of this receptor, possibly making carboxylated glycans the preferred S100-receptor. Finally, we show that TLR4 has a higher expression in isolated chondrocytes from OA patients than from non-OA patients. While this contradicts with findings from Zhang et al. (45), their study focused on intact femoral heads instead of isolated and cultured chondrocytes.

Coinciding with our results, chondrocytes from OA patients are more sensitive to S100A8 and S100A9 signaling, adding to the evidence that TLR4 is important in S100 signaling in primary human chondrocytes. Possibly, pro-inflammatory factors released by the activated synovium during OA may be responsible for upregulation of TLR4 and a higher sensitivity to TLR4-ligands such as S100A8 and S100A9.

Taken together, we conclude that it is very likely that TLR4 is the preferred receptor for S100A8 and S100A9 both in monocytes and macrophages as well as in the chondrocyte. The role of S100A8 and S100A9 in inflammation has

been established in recent years. However, its role in cartilage destruction is just starting to get unraveled. Cartilage damage is the most important feature in OA, with synovitis playing an increasingly significant role in the pathophysiology. This study links the effects of S100A8 and S100A9 as one of the most upregulated proteins during synovial activation to cartilage damage in OA and solidifies the potential of S100A8 and S100A9 as mediators of cartilage destruction in OA.

References

1. Goldring MB, Goldring SR. Osteoarthritis. J Cell Physiol 2007; 213(3):626-34.

2. van den Berg WB. Osteoarthritis year 2010 in review: pathomechanisms. Osteoarthritis Cartilage 2011.

3. Sellam J, Berenbaum F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nat Rev Rheumatol 2010; 6(11):625-35.

4. Attur M, Samuels J, Krasnokutsky S, Abramson SB. Targeting the synovial tissue for treating osteoarthritis (OA): where is the evidence? Best Pract Res Clin Rheumatol 2010; 24(1):71-9.

5. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis -- results of a 1 year longitudinal arthroscopic study in 422 patients. Osteoarthritis Cartilage 2005; 13(5):361-7.

6. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 2011; 7(1):33-42.

7. Bondeson J, Blom AB, Wainwright S, Hughes C, Caterson B, van den Berg WB. The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis. Arthritis Rheum 2010; 62(3):647-57.

8. Sunahori K, Yamamura M, Yamana J, Takasugi K, Kawashima M, Yamamoto H et al. The S100A8/A9 heterodimer amplifies proinflammatory cytokine production by macrophages via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase in rheumatoid arthritis. Arthritis Res Ther 2006; 8(3):R69.

9. Oppenheim JJ, Yang D. Alarmins: chemotactic activators of immune responses. Curr Opin Immunol 2005; 17(4):359-65.

10. Foell D, Wittkowski H, Vogl T, Roth J. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. J Leukoc Biol 2007; 81(1):28-37.

11. Marenholz I, Heizmann CW, Fritz G. S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophys Res Commun 2004; 322(4):1111-22.

12. Rammes A, Roth J, Goebeler M, Klempt M, Hartmann M, Sorg C. Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. J Biol Chem 1997; 272(14):9496-502.

13. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 2007; 13(9):1042-9.

14. van Lent PL, Grevers LC, Blom AB, Arntz OJ, van de Loo FA, van der Kraan P et al. Stimulation of chondrocyte-mediated cartilage destruction by S100A8 in experimental murine arthritis. Arthritis Rheum 2008; 58(12):3776-87.

15. Turovskaya O, Foell D, Sinha P, Vogl T, Newlin R, Nayak J et al. RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis. Carcinogenesis 2008; 29(10):2035-43.

16. Hammer HB, Odegard S, Syversen SW, Landewe R, van der HD, Uhlig T et al. Calprotectin (a major S100 leucocyte protein) predicts 10-year radiographic progression in patients with rheumatoid arthritis. Ann Rheum Dis 2010; 69(1):150-4.

17. Foell D, Roth J. Proinflammatory S100 proteins in arthritis and autoimmune disease.

Arthritis Rheum 2004; 50(12):3762-71.

18. Brun JG, Jonsson R, Haga HJ. Measurement of plasma calprotectin as an indicator of arthritis and disease activity in patients with inflammatory rheumatic diseases. J Rheumatol 1994; 21(4):733-8.

19. van Lent PL, Grevers L, Blom AB, Sloetjes A, Mort JS, Vogl T et al. Myeloid-related proteins S100A8/S100A9 regulate joint inflammation and cartilage destruction during antigen-induced arthritis. Ann Rheum Dis 2008; 67(12):1750-8.

20. Zreiqat H, Belluoccio D, Smith MM, Wilson R, Rowley LA, Jones K et al. S100A8 and S100A9 in experimental osteoarthritis. Arthritis Res Ther 2010; 12(1):R16.

21. Vogl T, Roth J, Sorg C, Hillenkamp F, Strupat K. Calcium-induced noncovalently linked tetramers of MRP8 and MRP14 detected by ultraviolet matrix-assisted laser desorption/ionization mass spectrometry. J Am Soc Mass Spectrom 1999; 10(11):1124-30.

22. Vogl T, Leukert N, Barczyk K, Strupat K, Roth J. Biophysical characterization of S100A8 and S100A9 in the absence and presence of bivalent cations. Biochim Biophys Acta 2006; 1763(11):1298-306.

23. Ii M, Matsunaga N, Hazeki K, Nakamura K, Takashima K, Seya T et al. A novel cyclohexene derivative, ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242), selectively inhibits toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling. Mol Pharmacol 2006; 69(4):1288-95.

24. Srikrishna G, Toomre DK, Manzi A, Panneerselvam K, Freeze HH, Varki A et al. A novel anionic modification of N-glycans on mammalian endothelial cells is recognized by activated neutrophils and modulates acute inflammatory responses. J Immunol 2001; 166(1):624-32.

25. van Lent PL, van den Berg WB, Schalkwijk J, van de Putte LB, van den BL. The impact of protein size and charge on its retention in articular cartilage. J Rheumatol 1987; 14(4):798-805.

26. Martel-Pelletier J, Boileau C, Pelletier JP, Roughley PJ. Cartilage in normal and osteoarthritis conditions. Best Pract Res Clin Rheumatol 2008; 22(2):351-84.

27. Cecil DL, Johnson K, Rediske J, Lotz M, Schmidt AM, Terkeltaub R. Inflammation-induced chondrocyte hypertrophy is driven by receptor for advanced glycation end products. J Immunol 2005; 175(12):8296-302.

28. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol 2009; 86(3):557-66.

29. Gebhardt C, Nemeth J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. Biochem Pharmacol 2006; 72(11):1622-31.

30. Abramson SB, Attur M. Developments in the scientific understanding of osteoarthritis. Arthritis Res Ther 2009; 11(3):227.

31. Smith MD, Triantafillou S, Parker A, Youssef PP, Coleman M. Synovial membrane inflammation and cytokine production in patients with early osteoarthritis. J Rheumatol 1997; 24(2):365-71.

32. Furuzawa-Carballeda J, ip-Rodriguez PM, Cabral AR. Osteoarthritis and rheumatoid arthritis pannus have similar qualitative metabolic characteristics and pro-inflammatory cytokine response. Clin Exp Rheumatol 2008; 26(4):554-60.

33. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis 2005; 64(9):1263-7.

34. Farahat MN, Yanni G, Poston R, Panayi GS. Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. Ann Rheum Dis 1993; 52(12):870-5.

35. Merz D, Liu R, Johnson K, Terkeltaub R. IL-8/CXCL8 and growth-related oncogene

alpha/CXCL1 induce chondrocyte hypertrophic differentiation. J Immunol 2003; 171(8):4406-15.

36. Ehlermann P, Eggers K, Bierhaus A, Most P, Weichenhan D, Greten J et al. Increased proinflammatory endothelial response to S100A8/A9 after preactivation through advanced glycation end products. Cardiovasc Diabetol 2006; 5:6.

37. van Lent PL, Grevers LC, Schelbergen R, Blom A, Geurts J, Sloetjes A et al. S100A8 causes a shift toward expression of activatory Fcgamma receptors on macrophages via toll-like receptor 4 and regulates Fcgamma receptor expression in synovium during chronic experimental arthritis. Arthritis Rheum 2010; 62(11):3353-64.

38. Miranda KJ, Loeser RF, Yammani RR. Sumoylation and nuclear translocation of S100A4 regulate IL-1beta-mediated production of matrix metalloproteinase-13. J Biol Chem 2010; 285(41):31517-24.

39. Cecil DL, Terkeltaub R. Transamidation by transglutaminase 2 transforms S100A11 calgranulin into a procatabolic cytokine for chondrocytes. J Immunol 2008; 180(12):8378-85.

40. Bobacz K, Sunk IG, Hofstaetter JG, Amoyo L, Toma CD, Akira S et al. Toll-like receptors and chondrocytes: the lipopolysaccharide-induced decrease in cartilage matrix synthesis is dependent on the presence of toll-like receptor 4 and antagonized by bone morphogenetic protein 7. Arthritis Rheum 2007; 56(6):1880-93.

41. Ghavami S, Rashedi I, Dattilo BM, Eshraghi M, Chazin WJ, Hashemi M et al. S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP kinase-dependent pathway. J Leukoc Biol 2008; 83(6):1484-92.

42. Boyd JH, Kan B, Roberts H, Wang Y, Walley KR. S100A8 and S100A9 mediate endotoxin-induced cardiomyocyte dysfunction via the receptor for advanced glycation end products. Circ Res 2008; 102(10):1239-46.

43. Yammani RR, Carlson CS, Bresnick AR, Loeser RF. Increase in production of matrix metalloproteinase 13 by human articular chondrocytes due to stimulation with S100A4: Role of the receptor for advanced glycation end products. Arthritis Rheum 2006; 54(9):2901-11.

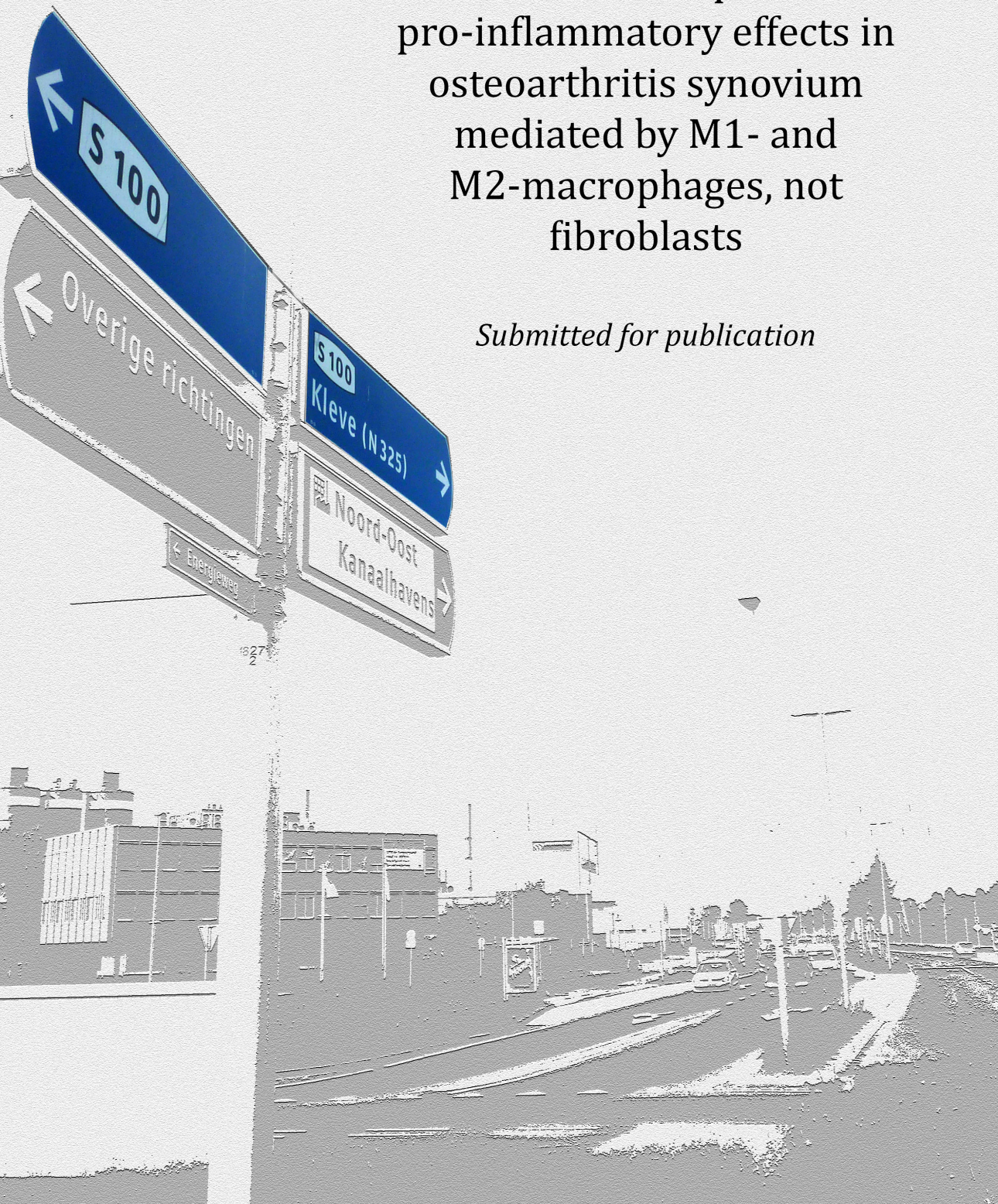
44. Srikrishna G, Panneerselvam K, Westphal V, Abraham V, Varki A, Freeze HH. Two proteins modulating transendothelial migration of leukocytes recognize novel carboxylated glycans on endothelial cells. J Immunol 2001; 166(7):4678-88.

45. Zhang Q, Hui W, Litherland GJ, Barter MJ, Davidson R, Darrah C et al. Differential Toll-like receptor-dependent collagenase expression in chondrocytes. Ann Rheum Dis 2008; 67(11):1633-41.

Chapter 5

Alarmin S100A9 promotes pro-inflammatory effects in osteoarthritis synovium mediated by M1- and M2-macrophages, not fibroblasts

Submitted for publication



Chapter 5

Alarmin S100A9 promotes pro-inflammatory effects in osteoarthritis synovium mediated by M1- and M2-macrophages, not fibroblasts.

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Submitted for publication

Abstract

Background: Alarmins S100A8/A9 have been shown to regulate synovial activation, cartilage damage and osteophyte formation in osteoarthritis (OA). In the current study we investigated the effect of S100A9 on OA synovium, M1- and M2-macrophages and OA fibroblasts.

Methods: End stage OA synovial explants, OA fibroblasts and human monocyte-derived M1- and M2-macrophages were stimulated with S100A9 and mRNA and protein levels of pro-inflammatory cytokines and MMPs were measured using RT-qPCR and Luminex.

Results: S100A9 upregulated pro-inflammatory cytokines IL-1β, -6, -8 and TNFα and MMP-9 in OA synovial explants ex vivo. However, in OA fibroblasts S100A9 had no significant effects on these mediators. S100A8/A9 mRNA and secreted protein was higher in M1- compared to M2-macrophages. Additional stimulation of M1-macrophages with S100A9 induced pro-inflammatory mediators IL-1β, -6, -8 on mRNA and IL-6 , -8 and TNFα on protein level and MMP-1 and -3 on mRNA. In M2-macrophages, S100A9 also upregulated these cytokines, yet to a lesser extent than in M1-macrophages. The mRNA level of TLR4 , the main receptor for S100A8/A9, was significantly lower in OA fibroblasts than in M1- and M2-macrophages

Conclusion: Alarmin S100A9 strongly upregulates pro-inflammatory and catabolic mediators in OA synovium and in both M1- and M2-macrophages, but not fibroblasts.

Introduction

Synovial inflammation is found in a large subgroup of osteoarthritis (OA) patients and it is believed to contribute to OA pathology (1, 2). The synovial intima layer consists of mainly two cell-types: the macrophage-like (type A) and fibroblast-like synoviocytes (type B). Although there are fewer synovial macrophages present in OA compared to rheumatoid arthritis (RA), they are crucial for the production of pro-inflammatory cytokines (such as IL-6 and IL-8) and cartilage matrix degrading enzymes, matrix metalloproteinases (MMP)-1 and -3 (3). Previous studies in our lab have shown that selective depletion of synovial macrophages during experimental OA largely reduces cartilage damage and osteophyte formation, two major hallmarks of OA (4, 5). Analogous to the Th1/Th2 nomenclature, classically activated and alternatively activated macrophages can be categorized as M1 and M2 macrophages respectively (6, 7). M1 macrophages can produce numerous pro-inflammatory cytokines such as TNFα and IL-1β and express MHC class II and CD86 receptors, while M2 macrophages produce anti-inflammatory cytokines IL-10 and IL-1RA and express scavenger receptor CD163 and mannose receptor (CD206) (7, 8).

Activation of the synovium during OA induces release of large amounts of alarmins S100A8 and S100A9 which can be easily measured in synovial fluid as well as in serum (9, 10). S100A8/A9 proteins are pro-inflammatory mediators produced by myeloid cells like monocytes and activated macrophages and have been shown to stimulate cells via TLR4. S100A8/A9 proteins exist as homodimers and heterodimers. The pro-inflammatory activity is particularly mediated by the homodimeric forms of S100A8 and S100A9. Earlier, we found that mice deficient for S100A9 (that also have no functional S100A8 protein) are largely protected against cartilage damage and synovial activation during collagenase induced OA (10), while also development of osteophytes was considerable less (Chapter 3). Furthermore, we showed that S100A8/A9 levels in the serum are elevated in early symptomatic OA patients and that high levels of these alarmins predict more cartilage damage and larger osteophytes at later stages in the disease (10)(Chapter 3). Earlier, we found that murine S100A8 induces release of TNFα in murine phagocytes (11) and human S100A8 and S100A9 were both able to induce a catabolic and pro-inflammatory phenotype in human OA chondrocytes,

in both cell-types via TLR-4 (12).

In the current study we analyzed S100A8/A9 production in M1- and M2-macrophages. Then we investigated the effects of S100A9 on the total cell population of human OA synovium and additionally zoomed in to specific effects of S100A9 on OA fibroblasts and macrophage subsets M1 and M2.

Materials and Methods

Human OA synovium

Permission of patients and the local ethics commission was obtained prior to harvesting of study samples. Human OA synovium was obtained from patients undergoing total knee or hip joint replacement surgery. Control synovium from patients with acute knee joint trauma was obtained at the time of arthroscopic examination.

Micro-array analysis

Preparation of complementary DNA and subsequent microarray analysis was done as described before (13). In short, generation of biotinylated complementary RNA, hybridization, and subsequent staining of MOE 430_2 oligonucleotide arrays (Affymetrix) was performed according to the Affymetrix Expression Analysis Technical Manual for 1-cycle amplification. Arrays were scanned using a laser scanner (GeneChip Scanner; Affymetrix) and analyzed using Affymetrix GeneChip Operating Software, version 1.4. Array normalization, expression value calculation, and clustering analysis were performed using DNA-Chip Analyzer (dChip) software, version 1.3 (online at www.dchip.org).

Immunohistochemistry and stimulations of human OA synovium

Synovium pieces of approximately 2x2 mm were cut from synovium obtained anonymously from end-stage OA patients undergoing arthroplasty. Pieces were randomized and 3 pieces/well were stimulated in quadruple for 24h with 1 µg/ml recombinant human (rh) S100A9 (developed in our labs (14)), other pieces

were fixed in formalin and afterwards embedded in paraffin. Tissue sections of 7 µm were cut, de-paraffinized and stained with mouse anti-human CD68 (clone KP1, Dako) and mouse anti-human CD163 (clone 10D6, Abcam).

Differentiation and stimulation of human M1- and M2-macrophages

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers after density gradient centrifugation of heparinized blood with Ficoll. Monocytes were isolated by magnetic-activated cell sorting (MACS) with specific CD14+ magnetic beads (Miltenyi Biotec). Monocytes were differentiated into M1 and M2 macrophages by adding 50 ng/ml rhGM-CSF or 20 ng/ml rhM-CSF (both R&D Systems), respectively, for 6 days with changing of the medium halfway. M1 and M2 macrophages were stimulated for 24h with 1 µg/ml rhS100A9 or 1 ng/ml rhIL-1β, after which cells were lysed in TRI-reagent (Sigma) for RNA isolation or collected in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) for flow cytometry. Supernatants were collected and stored at -20 °C to be measured for protein release by Luminex.

RNA isolation and RT-qPCR

RNA was isolated from OA fibroblasts and M1-M2 macrophages using TRI-reagent (Sigma-Aldrich), as described previously (15). OA synovium pieces were disrupted first with the MagNA Lyser (Roche) 5 times for 20 seconds at 6500 rpm and then RNA was isolated using the RNeasy-kit (Qiagen) according to the manufacturers protocol. RNA was reverse transcribed to cDNA and RT-qPCR was performed with specific primers and the SYBR Green Master mix in the StepOnePlus real-time PCR system (Applied Biosystems) as described before (15). Expression levels are expressed as minus delta Ct (ΔCt) values, normalized to the reference gene GAPDH.

Multiplex cytokine and matrix metalloproteinase protein measurement with Luminex

To determine protein levels of MMPs (MMP-1, MMP-3, MMP-9, MMP-13) and cytokines/chemokines (IL-6, IL-8 and TNFα) in culture medium, Luminex multianalyte technology on the Bio-Plex 100 system (Bio-Rad) was used in combination with multiplex MMP/cytokine kits (Milliplex from Millipore),

according to the Milliplex protocol.

S100A8/A9 proteins and ELISA

Recombinant human S100A9 were expressed and purified as previously described (14). Protein preparations were tested using the Limulus amoebocyte cell lysate (BioWhittaker), and lipopolysaccharide (LPS) content was undetectable (sensitivity 0.7 ± 0.5 pg LPS/μg protein), indicating that the maximal possible contamination of the S100 protein preparations was <1.2 pg LPS/μg protein. S100A9 lost activity while heat-inactivating at 80°C for 30 minutes. LPS activity was not changed at that temperature (11). S100A8/A9 concentrations were determined in supernatants of CD14+ monocytes and M1- and M2-macrophages by a sandwich enzyme-linked immunosorbent assay (ELISA) specifically for human S100A8/A9 as described previously (16).

Isolation and stimulation of human OA synovial fibroblasts

Human synovial fibroblasts originated from OA patients undergoing arthroplasty of knee or hip. OA synovium was isolated and tissue samples of approximately 2x2 mm were cultured for several weeks until adherent cells appeared. Adherent cells were considered fibroblasts on morphology. Non-adherent cells and tissue fragments were discarded. Fibroblasts were stimulated with 1 μg/ml S100A9 or with 1 ng/ml IL-1β for 24 hours. After stimulation, cells were lysed in TRI-reagent (Sigma) for mRNA measurement and supernatants were stored at -20°C for protein measurement.

Flow cytometry

S100A9-stimulated M2 macrophages were dissolved in PBS with 1% BSA and incubated with mouse anti-human CD163 (MAB1607, R&D Systems) followed by goat anti-mouse coupled with fluorescein isothiocyanate (FITC) (626312, Life Technologies) or with mouse anti-human CD206-FITC labelled (321104, Biolegend) only. Fluorescence was measured at 488 nm with the FACS Calibur (BD Bioscience) and analyzed using FlowJo software (TreeStar).

Statistics

Statistical differences were calculated with Students t-test, using Graph Pad Prism 5 (GraphPad Software). Differences were called significant with P-values < 0.05. Statistical analysis for array-based gene expression was performed using dChip software. The t statistic was computed as (mean1 - mean2)/√(SE(mean1)2 + SE(mean2)2); its value is calculated based on the t distribution, and the degree of freedom is set according to Welch modified two sample t test (17).

Results

Presence of M1 and M2 macrophages and expression of S100A8/A9 in OA synovium

Previously, we described that S100A8 and S100A9 mRNA were increased in OA synovium compared to normal synovium from patients in which acute joint trauma was suspected (11- and 10-fold, (10)). Synovial macrophages in OA express an activated phenotype but there is little known about their exact activation status (M1 versus M2). Now, we investigated gene expression of M1- and M2-markers in OA synovium using micro-array. For that, we used whole synovium explants from end-stage OA patients and healthy controls. Classical M2 markers CD163 and CD206 were present in total OA synovium, as well as anti-inflammatory M2 mediators IL-10 and IL-1RA (Table 1). CD206 and IL-10 were significantly upregulated in OA synovium (2.18 and 2.32 fold compared to healthy) while CD163 and IL-1RA were not significantly changed. M1 markers CD86, CD64 and TLR4 were also present in OA synovium, with CD86 being the only one significantly upregulated (1.8 fold) compared to healthy synovium (Table 1). These results indicate elevated presence of both M1 and M2 markers in OA synovium. Next, we confirmed the micro-array results with immunohistochemistry on paraffin-embedded sections of human OA synovium. We stained consecutive sections with CD68, a general macrophage marker and CD163, a specific M2 marker. CD68 was highly expressed in the synovial lining layer (Figure 1). Interestingly, in the same area there was also clear expression of CD163 (Figure 1). This suggests that there is a mixture of M2 and other possible more M1-like macrophages within the OA synovial lining layer.

	Fold change compared to healthy control	*p-value*	*Significance after correction for multiple testing*
M2-markers			
CD163	1,43 (-)	0,30	n.s.
CD206	2,18 (+)	0,02	*
IL-10	2,32 (+)	0,01	*
IL-1RA	2,1 (+)	0,04	n.s.
M1-markers			
CD86	1,8 (+)	0,03	*
CD64 (FcγRI)	1,73 (+)	0,36	n.s.
TLR4	1,67 (+)	0,05	n.s.

Table 1: Micro-array analysis of several M1- and M2-macrophage markers in OA synovium
Synovial RNA was isolated from end-stage OA patients undergoing arthroplasty of hip or knee and from healthy controls. Subsequent microarray analysis was performed on MOE 430_2 oligonucleotide array (Affymetrix). M1-macrophage markers CD86, CD64 (FcγRI) and TLR4 and M2-macrophage markers CD163, CD206, IL-10 and IL-1RA were compared to healthy synovium. Both M1- and M2-macrophage markers were all expressed in OA synovium. Of M2-markers, CD206 and IL-10 were significantly upregulated compared to healthy synovium, while of M1-markers only CD86 was significantly upregulated.

S100A8/A9 is expressed and secreted in high amounts by M1-macrophages and to a lesser extent by M2-macrophages

When entering the inflamed OA joint monocytes can differentiate into various macrophage phenotypes like pro-inflammatory M1 or anti-inflammatory M2, depending on the type of cytokines they meet within the synovium. While it is known that monocytes and activated macrophages produce and release S100A8/A9, nothing is known about the relative contribution of M1- and M2-macrophages. To investigate this, we differentiated human CD14+ monocytes into M1- or M2-macrophages and measured S100A8 and S100A9 mRNA levels and secreted S100A8/A9 protein and took along monocytes as positive control. Both S100A8 and S100A9 mRNA were significantly higher expressed in M1-macrophages compared to M2 (65.1 and 2.4-fold respectively) (Figure 2). In line with that, M1 macrophages produced significantly higher amounts of S100A8/

A9 protein compared to M2 (164% increase), and similar amounts of S100A8/A9 protein compared to monocytes (Figure 2).

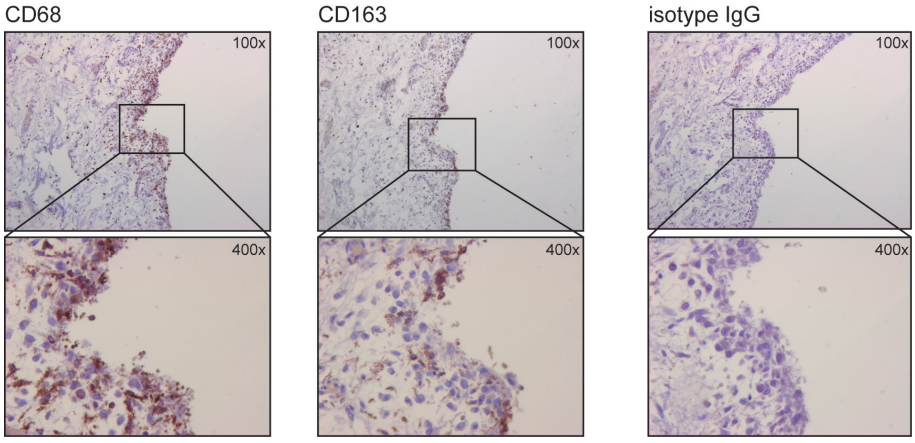

Figure 1: Expression of different macrophage markers in OA synovium
Synovial explants of end-stage OA patients undergoing arthroplasty of hip or knee were embedded in paraffin and tissue sections of 7 µm were cut. Consecutive sections were stained with specific antibodies against human CD68 (a common macrophage marker) or human CD163, a specific M2-macrophage marker or isotype IgG. Clear and abundant expression of CD68 was observed in mainly the lining layer of the OA synovium. CD163 was also clearly expressed in the lining layer of the synovium, suggesting presence of M2-macrophages.

S100A9 has pro-inflammatory effects on total OA synovium

S100A8 and S100A9 stimulate macrophages via TLR4 releasing additional S100 proteins and DAMPS, thereby forming a positive feedback-loop. Here, we studied whether S100A9 could stimulate OA synovium. We used S100A9 only, since we have shown before that it is the most potent human form and S100A8 and S100A9 show similar effects (12). Explants of whole OA synovium, containing fibroblasts and M1/M2 macrophages were stimulated ex vivo with human S100A9 and IL-1β as a positive control for 24h. S100A9 significantly induced mRNA expression of pro-inflammatory genes IL-1β, IL-6, IL-8 and TNFα (4.6, 2.7, 2.3 and 1.3 cycles compared to medium, respectively) and of catabolic MMP-9 (1.6 cycles), not of MMP-1, -3 and -13. (Figure 3A). Also, protein release of IL-6, IL-8 and TNFα was upregulated by S100A9 (9-, 14- and 22-fold increase respectively), while IL-1β protein could not be detected. S100A9 did not significantly change protein release of MMP-1, -3 and -9 (1.6-, 1.8- and 1.1-fold increase respectively). MMP-13 could not be detected in the supernatant (Figure 3B).

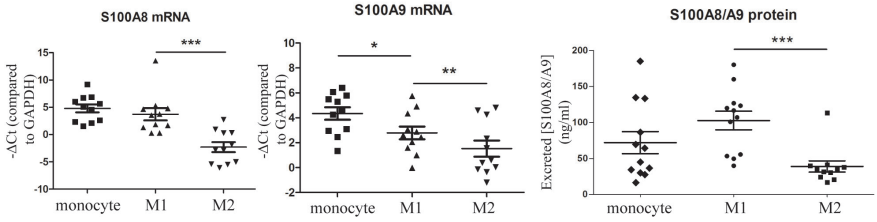

Figure 2: S100A8/A9 is expressed and released highly by M1-macrophages and to a lesser extent by M2-macrophages
Human monocytes were differentiated into M1- or M2-macrophages and mRNA expression and protein release of S100A8 and S100A9 were measured, also in the undifferentiated monocytes. S100A8 and S100A9 mRNA expression was significantly higher in M1- compared to M2-macrophages, while S100A9 mRNA expression in monocytes were even higher as in M1-macrophages. M1-macrophages release similar amounts of S100A8/A9 protein compared to monocytes yet significantly higher than M2-macrophages. N=11 donors. Every dot represent a separate donor. * = P<0.05, ** = P<0.01, *** = P<0.005 as measured by Students t-test.

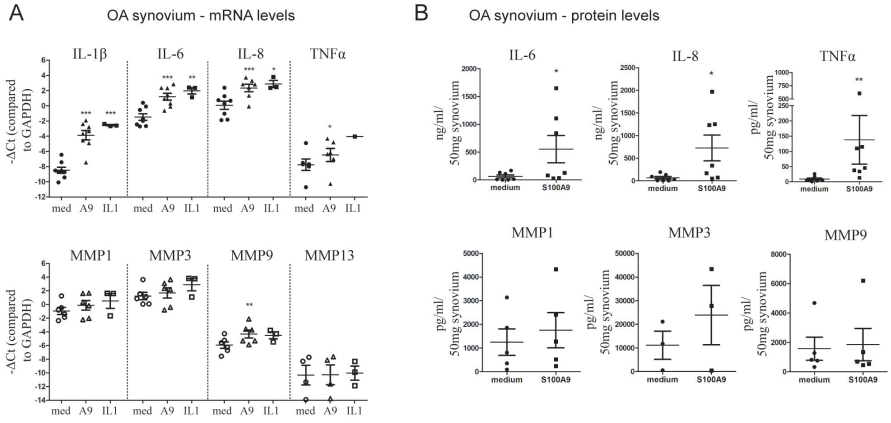

Figure 3: S100A9 increases pro-inflammatory mediators in OA synovium
Whole OA synovium from end-stage OA patients undergoing arthroplasty of hip or knee was stimulated ex vivo with S100A9 and IL-1β as a positive control. **A**. S100A9 significantly induced mRNA expression of pro-inflammatory genes IL-1β, IL-6, IL-8 and TNFα and of MMP-9, not of MMP-1, -3 and -13. **B**. Protein release of IL-6, IL-8 and TNFα was upregulated by S100A9, while IL-1β protein could not be detected. S100A9 did not significantly change protein release of MMP-1, -3 and -9. N=8 donors, although in some cytokine or MMP levels were below detection level. Every dot represent a separate donor. * = P<0.05, ** = P<0.01, *** = P<0.005 as measured by Students t-test. med=medium, A9=S100A9, IL1=IL-1β

S100A9 has no effects on OA fibroblasts

Next, we investigated whether synovial fibroblasts may significantly contribute to S100A9 upregulation of pro-inflammatory and catabolic factors in OA synovium. Fibroblasts were isolated from end-stage OA synovium by letting cells grow out and adhere to the plate for 1-2 weeks. Then we stimulated the fibroblasts with S100A9 (with IL-1β as positive control) for 24h, isolated mRNA and collected supernatants for protein measurement. No effect of S100A9 on mRNA expression of IL-1β, IL-6 and IL-8, nor on MMP-1 and -3 could be observed, while IL-1β strongly upregulated these genes in OA fibroblasts (Figure 4A). On protein level, S100A9 slightly upregulated IL-8 protein release, yet not significantly (2.4-fold increase), but not IL-6 (1.2-fold increase), while TNFα and IL-1β could not be detected (Figure 4B).

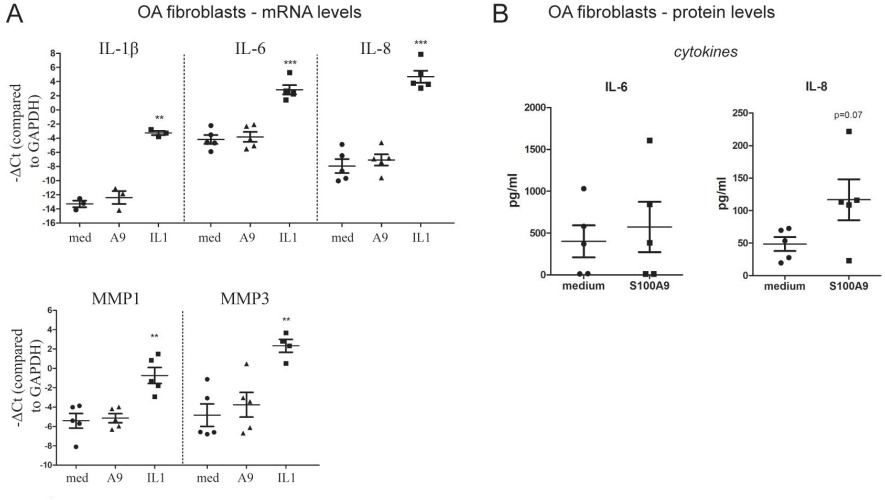

Figure 4: S100A9 has no significant effects on OA fibroblasts
OA fibroblasts were isolated from end-stage OA patients undergoing arthroplasty of hip or knee and stimulated with S100A9 and IL-1β as a positive control. **A**. On mRNA level, S100A9 did not change expression of cytokines IL-1β, IL-6 and IL-8, nor of MMP-1 and -3, while TNFα and MMP-9 and -13 were below detection level. In contrast, IL-1β significantly upregulated IL-1β, IL-6 and IL-8, and MMP-1 and -3. **B**. S100A9 also did not increase release of cytokines IL-6 and IL-8 by OA fibroblasts. IL-1β, TNFα, MMP-1, -3 and -9 could not be detected on protein level. N=5 donors, although in some cytokine or MMP levels were below detection level. Every dot represent a separate donor. * = P<0.05, ** = P<0.01, *** = P<0.005 as measured by Students t-test. med=medium, A9=S100A9, IL1=IL-1β

S100A9 has pro-inflammatory and catabolic effects on M1-macrophages

Next, we studied S100 stimulation of macrophages exhibiting different phenotypes. We differentiated human CD14+ monocytes into M1 or M2 macrophages and stimulated them with S100A9 for 24h, followed by measuring mRNA expression and protein release in the supernatant. S100A9 stimulation of M1 macrophages significantly upregulated mRNA levels of pro-inflammatory cytokines IL-1β, IL-6 and IL-8 (6.2, 3.8 and 4.4 cycles compared to medium, respectively), but not that of TNFα (0.1 cycles). Moreover, MMP-1 and -3 mRNA were also significantly upregulated (3.4 and 2.9 cycles compared to medium, respectively) (Figure 5A). On protein level, IL-6, IL-8 and TNFα were highly upregulated by S100A9 (90-, 33- and 167-fold increase), while IL-1β, MMP-1, -3 and -9 could not be detected on protein level (Figure 5B).

S100A9 has pro-inflammatory effects on regulatory M2-macrophages

Interestingly, S100A9 stimulation of the anti-inflammatory M2-macrophage also increased mRNA levels of IL-1β, IL-6 and IL-8 (5.0, 2.3 and 3.0 cycles compared to medium, respectively) but not TNFα (-0.2 cycles) (Figure 6A). Cytokine upregulation in M2 was slightly lower (1.2, 1.5 and 1.4 cycles respectively) than in M1-macrophages. In contrast to M1-macrophages, MMP-1 and MMP-3 mRNA were not upregulated in M2 (Figure 6A). On protein level, S100A9 also elevated release of pro-inflammatory cytokines IL-6, IL-8 and TNFα (41-, 67-, and 290-fold increase), although to considerably lower absolute amounts in M2 than in M1 macrophages (Figure 6B). Again, MMP-1, -3, and -9 protein levels were below detection level. We also measured mRNA expression of M1 and M2 receptors after S100A9 stimulation. Both M1 and M2 receptor markers CD86 / FcγR1 and CD163 / CD206 were not changed at the mRNA level, although a non-significant trend towards reduction of CD206 was observed (Figure 6C). Next, expression of M2 markers CD206 and CD163 was measured using flow cytometry. Expression of CD206 was significantly down-regulated by S100A9, both on the total percentage of positive cells as on the mean fluorescence intensity (MFI). Protein expression of CD163, another classical M2-marker, was however not affected by S100A9 stimulation of M2 macrophages (Figure 6D). Finally, we investigated why S100A9 stimulation may be more potent on macrophage subtypes M1 and M2

than on fibroblasts. As TLR-4 has earlier been shown to be the main receptor for signaling S100A8/A9 (11, 12, 18), we measured basal mRNA expression of TLR-4 on OA fibroblasts, M1- and M2-macrophages. TLR-4 mRNA was significantly higher in both M1- and M2-macrophages, compared to fibroblasts (2.5 and 3.5 cycles, respectively), while there was no significant difference in TLR-4 expression between M1- and M2-macrophages (Figure 6E).

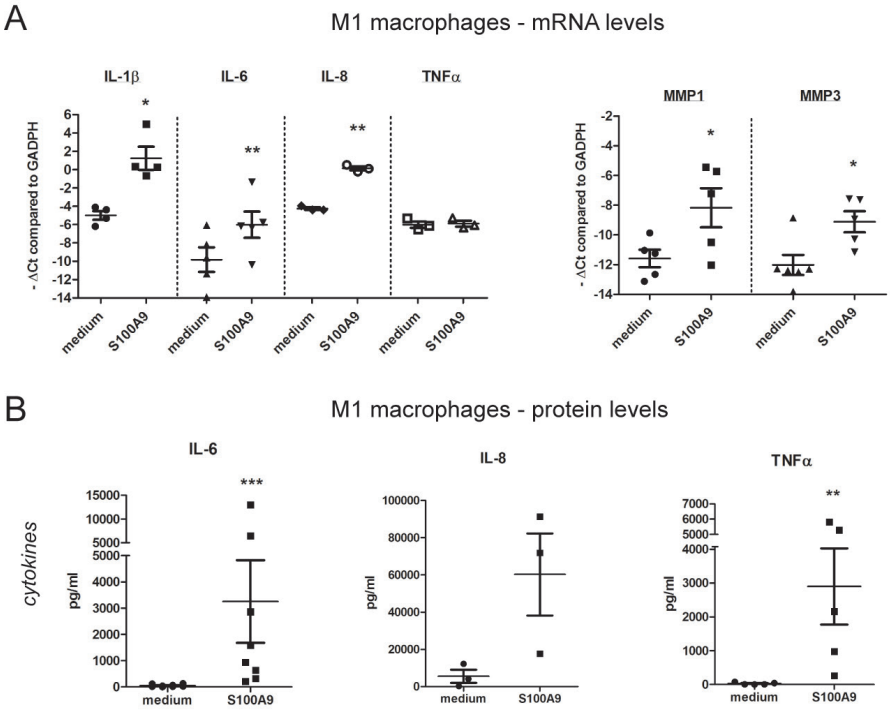

Figure 5: S100A9 increases pro-inflammatory and catabolic mediators in M1-macrophages
Human CD14+ monocytes were differentiated into M1-macrophages with GM-CSF and stimulated with S100A9 for 24h, followed by measuring mRNA expression (**A**) and protein release (**B**). **A**. S100A9 significantly upregulated mRNA expression of pro-inflammatory cytokines IL-1β, IL-6 and IL-8, not TNFα. Also mRNA of MMP-1 and -3 were upregulated by S100A9. **B**. On protein level, S100A9 significantly upregulated cytokine release of IL-6 and TNFα. IL-8 protein release was increased by S100A9, however this did not reach significance. IL-1β, MMP-1, -3 and -9 could not be detected on protein level. N=5 donors in **A**, n=8 in **B**, although in some cytokine or MMP levels were below detection level. Every dot represent a separate donor. * = P<0.05, ** = P<0.01, *** = P<0.005 as measured by Students t-test.

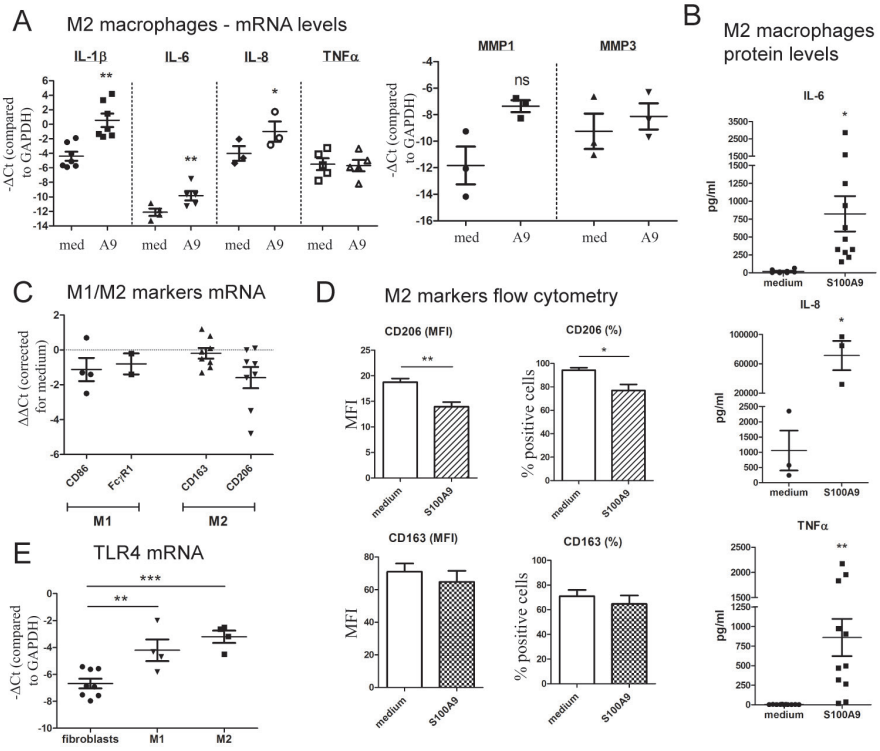

Figure 6: S100A9 has pro-inflammatory effects on M2-macrophages
Human CD14+ monocytes were differentiated into M2-macrophages with M-CSF and stimulated with S100A9 for 24h, followed by measuring mRNA expression (**A-C**), protein release (**B**) or protein expression on the membrane (**D**). **A**. S100A9 significantly upregulated mRNA expression of pro-inflammatory cytokines IL-1β, IL-6 and IL-8, not TNFα, in M2-macrophages. mRNA expression of MMP-1 and -3 were not upregulated by S100A9 in M2-macrophages. **B**. On protein level, S100A9 significantly upregulated cytokine release of IL-6, IL-8 and TNFα by M2-macrophages, although the absolute levels reached were lower as by M1-macrophages. **C**. S100A9 stimulation of M2-macrophages did not significantly change mRNA expression of M1- and M2-macrophage markers CD86, FCγRI (CD64), CD163 and CD206. **D**. As measured by flow cytometry, S100A9 stimulation of M2-macrophages downregulated M2-marker CD206 on Median Fluorescence Intensity (MFI) and total percentage of positive cells. M2-marker CD163 was not changed on the membrane of M2-macrophages by S100A9 stimulation. **E**. mRNA levels of TLR4, described receptor for S100A9, was higher in M1- and M2-macrophages compared to OA fibroblasts. N=8 donors in **A** and **C**, n=10 in **B**, n=3 in **D** and variable in **E**. Every dot represent a separate donor. * = P<0.05, ** = P<0.01, *** = P<0.005 as measured by Students t-test. med=medium, A9=S100A9

Discussion

In the current study we show that alarmins S100A8/A9 can upregulate pro-inflammatory and catabolic mediators in OA synovium ex vivo. Moreover, S100A9 can induce pro-inflammatory and catabolic mediators not only in pro-inflammatory M1 type macrophages, but interestingly also in regulatory M2 macrophages, inducing an intermediate M1/M2 macrophage phenotype.

In this study micro-array analysis of OA synovium showed presence of both M1- and M2-macrophage markers, while we could confirm the expression of the M2 marker CD163 in OA synovium using immunohistochemistry. CD86 and CD206 can also be expressed by other immune cells like dendritic cells and B-cells. However, involvement and presence of these cells in OA is very low. Moreover, Fahy et al. recently showed combined presence of CD86 (M1 marker) and CD206 (M2 marker) in OA synovium by histology (19). In spondyloarthritis and rheumatoid arthritis both types are equally present as well (20). Together with our data, this suggests presence of both M1- and M2-macrophages in arthritic synovium.

From several studies it seems clear that macrophages in OA exhibit an activated phenotype, producing amongst others IL-6, IL-8 and vascular endothelial growth factor (VEGF). Moreover, OA synovial cell cultures depleted of macrophages no longer produce IL-1β and TNFα (3, 21) suggesting that the macrophage is the major cell type regulating the release of pro-inflammatory factors in OA synovial activation. From experimental OA models we know that synovial inflammation and IL-1β, TNFα and IL-6 are high only at the start of OA, while waning at later time-points (10). In contrast, S100A8 and S100A9 stay high during the course of the model. Seen in this light, M1-macrophages could well be dominant in the first phase, while M2-macrophages take over in the end stages of the disease. Both cell phenotypes however can produce S100A8 and S100A9 and become strongly activated after addition of these proteins as we have shown in the current study. By this mechanism, S100A8 and S100A9 can perpetuate synovial inflammation throughout the disease course of OA.

In our study, we show that S100A8 and S100A9 are preferentially expressed and

produced by M1-macrophages, even as high as monocytes, known to be potent producers of S100 (22). In line with our results, a recent mouse study by Dessing et al. shows that also murine bone-marrow derived M1-macrophages express more S100A8 and S100A9 mRNA than M2. Moreover, they show that S100A9 -/- bone marrow cells preferentially differentiate into arginase-1 positive M2-type macrophages, suggesting absence of S100-proteins skews macrophages to an M2-phenotype (23).

We also show that S100A9 acts most potently on M1-macrophages, but is also a strong stimulator yet slightly less potent, of M2-macrophages, while there is no significant effect of S100A9 on synovial fibroblasts. This difference in potency might be due to the expression levels of TLR4, as we show by qPCR. In line with this, we showed earlier that upregulation of TNFα in monocytes by S100A8 is TLR4-dependent (11) and that upregulation of Fcγ receptors by S100A8 was completely absent in TLR4 -/- macrophages (24). Moreover, we demonstrated that TLR4 also mediated S100A8/A9 effects in both chondrocytes and osteoclasts (12, 18). Some studies report the interaction of S100A8 and/or S100A9 with RAGE (25), yet signaling through RAGE is only reported for other S100 family members S100A11 and S100A4 (26, 27). TLR4 expression in synovial fibroblasts however is not completely absent, and S100A8/A9 could still have some effects. This could be due to the different signaling pathways of TLR4. Recently, we found that TLR4 signaling in fibroblasts is specifically TGFβ-activated kinase (TAK1) independent (28), while TAK1 is a key regulator of TLR mediated activation of NFκB and mitogen activated protein kinase (MAPK) pathways in macrophages. Moreover, S100A8 and S100A9 have been shown to act via the TAK1-NFκB pathway (29). Possibly, TLR4 signaling through the MyD88-TAK1 dependent pathway might be less dominant or even ablated in fibroblasts.

Interestingly we found that stimulation of M2-macrophages with S100A9 upregulated pro-inflammatory cytokines and down-regulated M2 marker CD206, while CD163, another well-known M2 marker, was not changed. Earlier studies show that skewing of M2- to M1-type myeloid-derived cells can be induced with LPS, which also acts via TLR4 (30). Another factor that has been described to skew M2 towards M1 is oxidized low-density lipoprotein (LDL) inducing pro-inflammatory cytokines IL-6, -8 and MCP-1 in M2-macrophages (31). Interestingly, we showed earlier that (oxidized) LDL increases S100A8 expression in synovial macrophages (15), suggesting that the effect of (oxidized)

LDL runs via S100A8/A9. M1 and M2 are macrophage phenotypes at the opposite sides of a spectrum, with multiple intermediate stages. The group of Schultze differentiated human M1 and M2 macrophages with GM-CSF and M-CSF (similar to our approach) and consequently stimulated these with 28 different stimuli. When analyzing the transcriptome, they found that macrophages have the ability to respond specifically and differently to each stimulus (32). Apparently, also S100A9 skews the M2-macrophage towards a yet undefined pro-inflammatory macrophage.

Taken together, our study indicates that S100A8/A9 can induce and perpetuate a pro-inflammatory loop in OA synovium via M1-macrophages and to a lesser extent M2-macrophages. Considering the long-time expression of S100A8/A9 in the synovium during OA, targeting these proteins could be an interesting option for future therapies of OA.

Acknowledgments

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References

1. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis -- results of a 1 year longitudinal arthroscopic study in 422 patients. Osteoarthritis Cartilage. 2005;13(5):361-7.

2. Sokolove J, Lepus CM. Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. Ther Adv Musculoskelet Dis. 2013;5(2):77-94.

3. Bondeson J, Blom AB, Wainwright S, Hughes C, Caterson B, van den Berg WB. The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis. Arthritis Rheum. 2010;62(3):647-57.

4. Blom AB, van Lent PLEM, Holthuysen AEM, van der Kraan PM, Roth J, van Rooijen N, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. Osteoarthritis Cartilage. 2004;12(8):627-35.

5. Blom AB, van Lent PL, Libregts S, Holthuysen AE, van der Kraan PM, van Rooijen N, et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. Arthritis Rheum. 2007;56(1):147-57.

6. Gordon S. Alternative activation of macrophages. Nature reviews Immunology. 2003;3(1):23-35.

7. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004;25(12):677-86.

8. Ambarus CA, Krausz S, van Eijk M, Hamann J, Radstake TR, Reedquist KA, et al. Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. Journal of immunological methods. 2012;375(1-2):196-206.

9. Sunahori K, Yamamura M, Yamana J, Takasugi K, Kawashima M, Yamamoto H, et al. The S100A8/A9 heterodimer amplifies proinflammatory cytokine production by macrophages via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase in rheumatoid arthritis. Arthritis Res Ther. 2006;8(3):R69.

10. van Lent PLEM, Blom AB, Schelbergen RFP, Sloetjes A, Lafeber FPJG, Lems WF, et al. Active involvement of alarmins S100A8 and S100A9 in the regulation of synovial activation and joint destruction during mouse and human osteoarthritis. Arthritis Rheum. 2012;64(5):1466-76.

11. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007;13(9):1042-9.

12. Schelbergen RFP, Blom AB, van den Bosch MHJ, Sloetjes A, Abdollahi-Roodsaz S, Schreurs BW, et al. Alarmins S100A8 and S100A9 elicit a catabolic effect in human osteoarthritic chondrocytes that is dependent on Toll-like receptor 4. Arthritis Rheum. 2012;64(5):1477-87.

13. Takahashi N, de Jager VC, Gluck A, Letzkus M, Hartmann N, Staedtler F, et al. The molecular signature of oxidative metabolism and the mode of macrophage activation determine the shift from acute to chronic disease in experimental arthritis: critical role of interleukin-12p40. Arthritis Rheum. 2008;58(11):3471-84.

14. Vogl T, Leukert N, Barczyk K, Strupat K, Roth J. Biophysical characterization of S100A8 and S100A9 in the absence and presence of bivalent cations. Biochim Biophys Acta. 2006;1763(11):1298-306.

15. de Munter W, Blom AB, Helsen MM, Walgreen B, van der Kraan PM, Joosten LA,

et al. Cholesterol accumulation caused by low density lipoprotein receptor deficiency or a cholesterol-rich diet results in ectopic bone formation during experimental osteoarthritis. Arthritis Res Ther. 2013;15(6):R178.

16. Wulffraat NM, Haas PJ, Frosch M, De Kleer IM, Vogl T, Brinkman DMC, et al. Myeloid related protein 8 and 14 secretion reflects phagocyte activation and correlates with disease activity in juvenile idiopathic arthritis treated with autologous stem cell transplantation. Ann Rheum Dis. 2003;62(3):236-41.

17. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A. 2001;98(1):31-6.

18. Grevers LC, de Vries TJ, Vogl T, Abdollahi-Roodsaz S, Sloetjes AW, Leenen PJM, et al. S100A8 enhances osteoclastic bone resorption in vitro through activation of Toll-like receptor 4: implications for bone destruction in murine antigen-induced arthritis. Arthritis Rheum. 2011;63(5):1365-75.

19. Fahy N, de Vries-van Melle ML, Lehmann J, Wei W, Grotenhuis N, Farrell E, et al. Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. Osteoarthritis Cartilage. 2014.

20. Ambarus CA, Noordenbos T, de Hair MJ, Tak PP, Baeten DL. Intimal lining layer macrophages but not synovial sublining macrophages display an IL-10 polarized-like phenotype in chronic synovitis. Arthritis Res Ther. 2012;14(2):R74.

21. Bondeson J. Activated synovial macrophages as targets for osteoarthritis drug therapy. Curr Drug Targets. 2010;11(5):576-85.

22. Rammes A, Roth J, Goebeler M, Klempt M, Hartmann M, Sorg C. Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. J Biol Chem. 1997;272(14):9496-502.

23. Dessing MC, Tammaro A, Pulskens WP, Teske GJ, Butter LM, Claessen N, et al. The calcium-binding protein complex S100A8/A9 has a crucial role in controlling macrophage-mediated renal repair following ischemia/reperfusion. Kidney international. 2014.

24. van Lent PL, Grevers LC, Schelbergen R, Blom A, Geurts J, Sloetjes A, et al. S100A8 causes a shift toward expression of activatory Fcgamma receptors on macrophages via toll-like receptor 4 and regulates Fcgamma receptor expression in synovium during chronic experimental arthritis. Arthritis Rheum. 2010;62(11):3353-64.

25. Ghavami S, Rashedi I, Dattilo BM, Eshraghi M, Chazin WJ, Hashemi M, et al. S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP kinase-dependent pathway. J Leukoc Biol. 2008;83(6):1484-92.

26. Cecil DL, Johnson K, Rediske J, Lotz M, Schmidt AM, Terkeltaub R. Inflammation-induced chondrocyte hypertrophy is driven by receptor for advanced glycation end products. J Immunol. 2005;175(12):8296-302.

27. Yammani RR, Carlson CS, Bresnick AR, Loeser RF. Increase in production of matrix metalloproteinase 13 by human articular chondrocytes due to stimulation with S100A4: Role of the receptor for advanced glycation end products. Arthritis Rheum. 2006;54(9):2901-11.

28. Geurts J, van den Brand BT, Wolf A, Abdollahi-Roodsaz S, Arntz OJ, Kracht M, et al. Toll-like receptor 4 signalling is specifically TGF-beta-activated kinase 1 independent in synovial fibroblasts. Rheumatology (Oxford). 2011;50(7):1216-25.

29. Kang JH, Hwang SM, Chung IY. S100A8, S100A9, and S100A12 activate airway epithelial cells to produce MUC5AC via ERK and NF-kappaB pathways. Immunology. 2014.

30. Yang Y, Zhang R, Xia F, Zou T, Huang A, Xiong S, et al. LPS converts Gr-1(+) CD115(+) myeloid-derived suppressor cells from M2 to M1 via P38 MAPK. Exp Cell Res.

2013;319(12):1774-83.

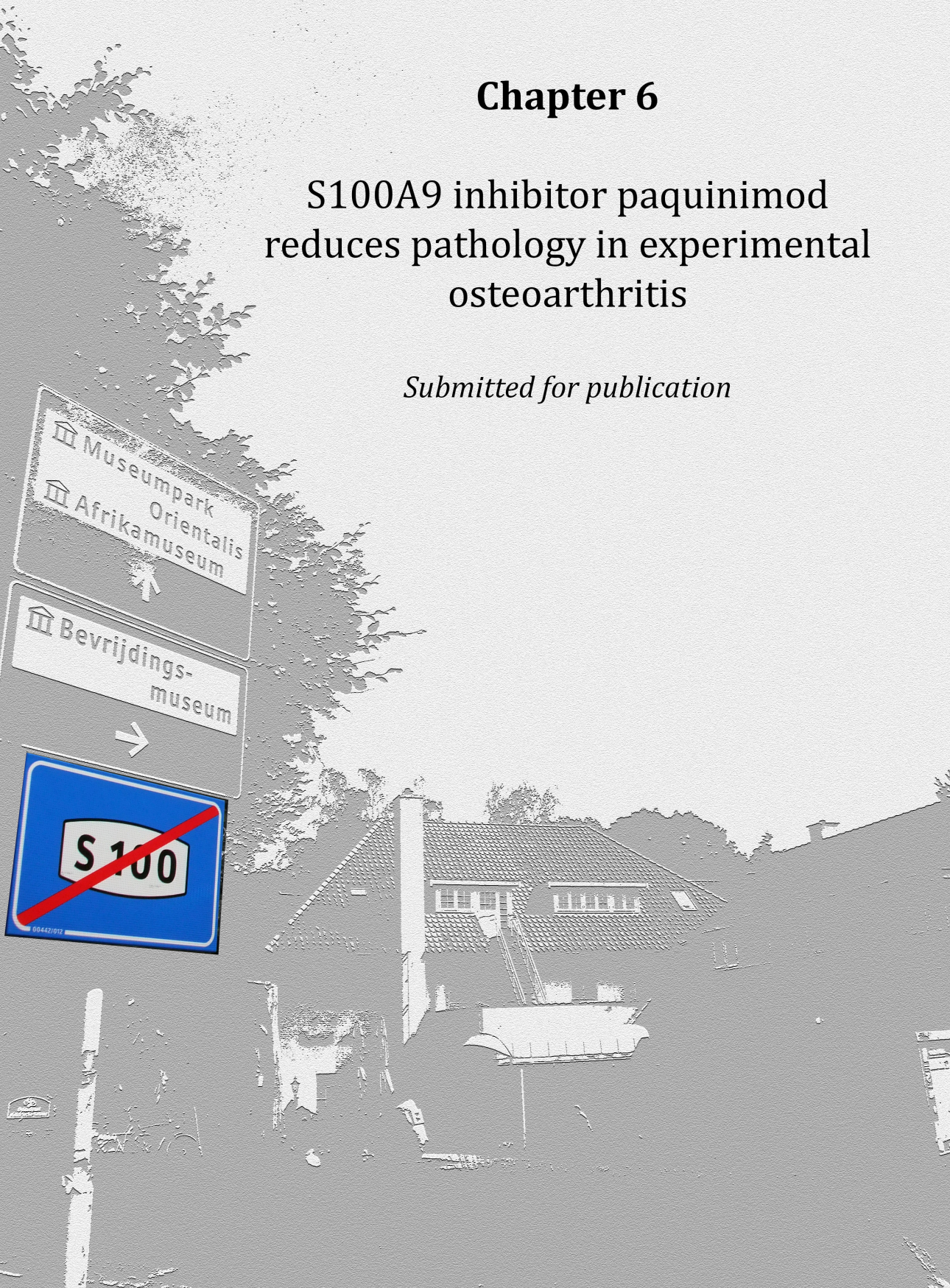
31. van Tits LJ, Stienstra R, van Lent PL, Netea MG, Joosten LA, Stalenhoef AF. Oxidized LDL enhances pro-inflammatory responses of alternatively activated M2 macrophages: a crucial role for Kruppel-like factor 2. Atherosclerosis. 2011;214(2):345-9.

32. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. Immunity. 2014;40(2):274-88.

Chapter 6

S100A9 inhibitor paquinimod reduces pathology in experimental osteoarthritis

Submitted for publication



Chapter 6

S100A9 inhibitor paquinimod reduces pathology in experimental osteoarthritis

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Abstract

Objectives: Alarmins S100A8/A9 regulate pathology in experimental osteoarthritis (OA). Paquinimod is an immunomodulatory compound preventing S100A9 binding to TLR-4. We investigated the effect of paquinimod on experimental OA and human OA synovium.

Materials and Methods: Two OA mouse models differing in level of synovial activation were treated with paquinimod. Synovial thickening, osteophyte size and cartilage damage were measured histologically, using an arbitrary score, adapted Pritzker OARSI score or imaging software, respectively. Human OA synovia were stimulated with S100A9, with or without paquinimod.

Results: Paquinimod treatment of collagenase-induced OA (CIOA) resulted in significantly reduced synovial thickening (57%), osteophyte size at the medial femur (66%) and cruciate ligaments (67%) and cartilage damage at the medial tibia (47%) and femur (75%). In contrast, paquinimod did not reduce cartilage damage and reduced osteophyte size only slightly in DMM (destabilization of the medial meniscus), an OA model with considerably lower synovial activation compared to CIOA. In human OA synovium, paquinimod blocked pro-inflammatory (IL-6, IL-8, TNFα) and catabolic (MMP-1 and -3) factors induced by S100A9.

Conclusions: Paquinimod reduces synovial activation, osteophyte formation and cartilage damage in experimental OA with high synovial activation (CIOA) and can block pathological effects of S100A9 in OA synovium *ex vivo*.

Introduction

Osteoarthritis (OA), one of the ten most disabling diseases in developed countries, has no effective therapy to date. Although historically considered a disease of the cartilage only, it is now clear that other tissues like the synovium also play an important role in the pathophysiology of OA (1). Synovial activation is present in at least 50% of OA patients (2) and several pro-inflammatory mediators have been shown to be involved in OA pathology (3, 4), among which alarmins S100A8 and S100A9. In recent studies, we have shown that S100A8/A9 is high in serum and synovium of OA patients and predictive for cartilage damage and osteophyte formation (Chapter 3 and (5)). S100A9 -/- mice show reduced joint pathology in collagenase-induced OA (CIOA), a model with considerable synovitis. However, no effect of the absence of S100A9 was seen in the surgical, non-inflammatory DMM model (destabilization of the medial meniscus) (5). S100A8 and S100A9 are endogenous ligands for TLR-4 (6) and blocking this interaction in OA chondrocytes reduces the catabolic effects of these alarmins (7). Quinoline-3-carboxamide paquinimod (ABR-215757) is effective in experimental lupus and encephalomyelitis and proven to be safe and well tolerated in SLE patients (8, 9). Paquinimod binds with high affinity to both human and mouse S100A9 and specifically blocks the interaction of S100A9 to its receptors TLR-4 and RAGE (10).

In the current study we investigated the effect of the S100A9-blocking compound paquinimod on experimental osteoarthritis with different degrees of synovial activation. Furthermore, we tested the in vitro effects of paquinimod on human OA synovial tissue.

Material and Methods

Human OA synovium preparation and stimulation

Human synovium was obtained anonymously from OA patients undergoing arthroplasty. Synovium pieces (2x2 mm) were randomized and 3 pieces/

well were cultured and stimulated in quadruplo for 24h with 1μg/ml human S100A9, 350 μM paquinimod or both (30 minutes pre-incubated). Thereafter, synovium pieces were weighed and fixed in formalin and embedded in paraffin for histological analysis with hematoxylin-eosin (HE) staining and with a specific mouse anti-human CD68 (clone KP1, Dako). OA synovium supernatant was collected for protein measurement on the Bio-Plex 100 Luminex system (Bio-rad).

Animals and animal models

Experimental OA was elicited in 12-14 weeks old male C57BL/6J (Janvier) and S100A9-/- in a C57BL/6 background. Mice were housed in groups of 10 animals in filter-top cages, and water and food were provided ad libitum. Collagenase-induced osteoarthritis (CIOA) was performed by 2x 1U collagenase (C0773, Sigma) injection into the knee-joint (11). Destabilized medial meniscus (DMM) OA was induced by transection of the medial anterior meniscotibial ligament (12). All animal experiments were approved by the local authority Animal Care and Use Committee and local ethics committee (RU-DEC 2012-232) and were performed by personnel certified by the Dutch Ministry of WVC.

Paquinimod preparation and administration

Paquinimod (ABR-215757) was synthesized by Active Biotech AB and administered in the drinking water of mice to a final concentration of 3,75 mg/kg. Normal tap water in which the paquinimod was dissolved served as control.

Histological analysis

Total knee joints were fixed in formalin, embedded in paraffin and cut in 7 μm sections. Osteophyte size was assessed in Safranin-O/Fast Green stained sections using the Leica Application Suite (LAS) image analysis software. Cartilage damage was measured blinded using an arbitrary score based on the OARSI scoring method by Pritzker et al. (13). Briefly, lateral and medial tibia and femur were scored for the grade of cartilage destruction (0-5) and the extent of damaged cartilage surface (0-6). These scores were multiplied and added together for 5 sections to form the total OA cartilage score. Synovial thickening/activation was scored on HE stained paraffin sections using an arbitrary score from 0-3 as

previously described (14).

Statistical analysis

Statistical differences were calculated with student t-test or Mann Whitney U as indicated using Graph Pad Prism 5 (GraphPad Software).

Results

Paquinimod reduces synovial activation, osteophyte formation and cartilage damage in collagenase induced OA

We treated experimental OA with high (CIOA) and low synovial activation (DMM model) with paquinimod, administered via the drinking water. Treatment started 4 days prior to induction of either model and was refreshed twice a week. Paquinimod was well tolerated and caused no apparent side effects in both models. Control- and paquinimod-treated animals did not differ in weight when measured on day 11 (weight increase control vs. paquinimod in CIOA 105% vs. 106%, in DMM 114% vs. 114%) nor on day 42 (weight increase control vs. paquinimod in CIOA 117% vs. 117%, in DMM 117% vs. 115%) (data not shown), indicating that drinking behavior was not affected.

CIOA was treated with paquinimod and on day 42 we measured synovial thickening, osteophyte formation and cartilage damage, three important hallmarks of OA pathology. Synovial thickening was significantly reduced by paquinimod-treatment at the medial side of the patella-femur region (57%) (Figure 1A). Osteophyte size was significantly reduced at the medial femur (66%) and cruciate ligament (67%) (Figure 1B). A trend towards reduction was also observed in the medial collateral ligament and in total osteophyte size, but this did not reach significance. Finally, cartilage damage was significantly reduced by paquinimod treatment of CIOA at both medial tibia and femur (47% and 75% respectively) and in the total joint score (47% reduction) (Figure 1C).

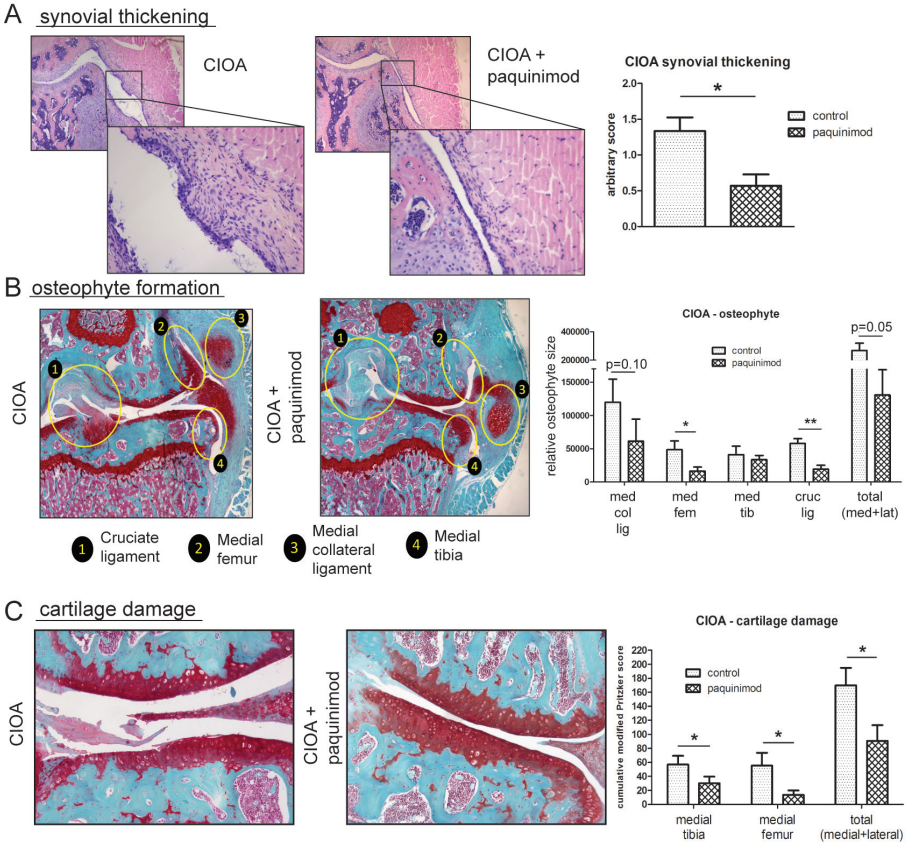

Figure 1: Treatment of collagenase induced OA with paquinimod significantly reduced synovial thickening, osteophyte formation and cartilage damage.
Collagenase-induced osteoarthritis (CIOA) was induced by two times collagenase injection into the knee joint of C57BL/6J mice, an experimental OA model with high synovial activation. We treated the mice starting four days prior to induction of OA with 3,75 mg/kg paquinimod and assessed OA pathology on day 42. **A**. Synovial thickening, scored on an arbitrary scale from 0-3 on hematoxylin/eosin (HE) stained histological slides, was significantly reduced by paquinimod at the medial side of the femur. **B**. Osteophyte size was measured with image analysis software on Safranin-O/Fast Green stained histological slides. Paquinimod reduced osteophyte size on day 42 of CIOA at the medial femur and cruciate ligament, while a trend towards reduction was observed at the medial collateral ligament and in the total medial and lateral osteophyte size. med=medial, col=collateral, lig=ligament, fem=femur, tib=tibia, cruc=cruciate, lat=lateral **C**. Cartilage damage was assessed on Safranin-O/Fast Green stained histological slides with the modified Pritzker OARSI score. Paquinimod significantly reduced cartilage damage on day 42 of CIOA at the medial tibia and femur and in the total joint. * = P<0.05, ** = P<0.01 as measured by Mann Whitney test (**A**) or student t-test (in **B**, **C**).

Paquinimod has marginal effects in DMM

Paquinimod treatment of DMM OA, in which synovial activation is scant, did not reduce osteophyte size in neither medial tibia nor femur (Figure 2A). Cartilage damage was significantly reduced by paquinimod treatment only at the medial femur (64%), but not at the medial tibia where most damage occurs in DMM (Figure 2B).

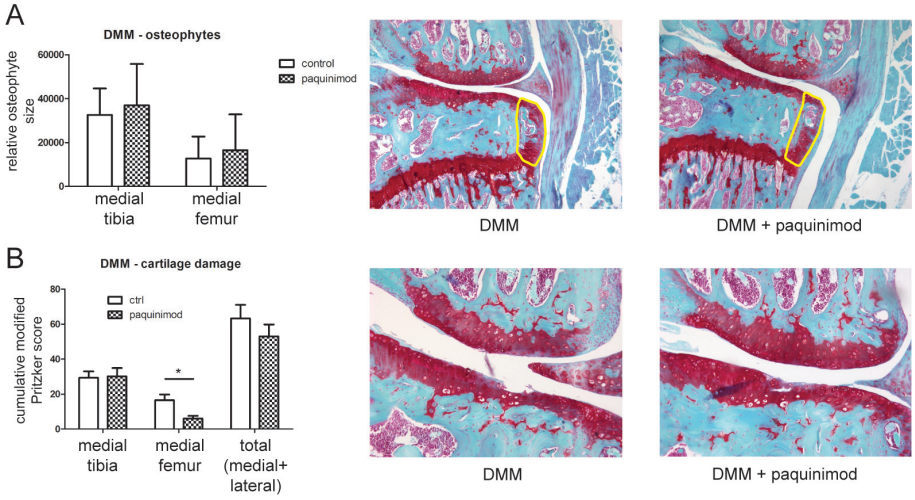

Figure 2: Paquinimod does not reduce osteophyte size and only marginally reduces cartilage damage in surgically induced experimental OA.
DMM (destabilization of the medial meniscus) was surgically induced in C57BL/6J mice and treatment with 3,75 mg/kg paquinimod was started four days prior. Synovial activation in the DMM model is low and cartilage damage and osteophyte formation occur mostly at the medial femur and tibia. Osteophyte size and cartilage damage were assessed on day 56 **A**. Paquinimod did not reduce osteophyte size on day 56 of DMM at medial tibia or femur, as measured with image analysis software on Safranin-O/Fast Green stained histological slides. **B**. Cartilage damage was assessed on Safranin-O/Fast Green stained histological slides with the modified Pritzker OARSI score. Paquinimod significantly reduced cartilage damage at the medial femur on day 56 of DMM, however not at the medial tibia where most damage occurs. * = P<0.05 measured by student t-test.

Paquinimod reduces pro-inflammatory/catabolic effects in vitro in macrophages and OA synovial tissue

To test paquinimod in a human OA setting, we investigated the effect of the compound on synovial specimen obtained after joint replacement of end-stage OA patients. Macroscopically, we excluded non-synovial tissue like cartilage, tendon and fat and we could confirm this on histology using HE staining. We

stained for CD68 demonstrating presence of macrophages (Figure 3A). Then we incubated OA synovium with human S100A9 (15) and/or paquinimod, and measured the protein release. Addition of paquinimod alone did not have an effect on pro-inflammatory cytokines TNFα, IL-6 and IL-8 nor on matrix metalloproteinases (MMP)-1, -3 and -9 (IL-1β and MMP-13 were not detectable) (Figure 3B). S100A9 alone clearly upregulated IL-6, IL-8 and TNFα protein levels in OA synovia compared to medium control (9-fold, 12-fold and 20-fold increase respectively), and addition of paquinimod significantly inhibited this upregulation for IL-6 (35% reduction) and IL-8 (38% reduction), but not TNFα (Figure 3C). S100A9 significantly upregulated MMP-1 and MMP-3 (up to 2,5 fold) and this catabolic effect could be partially inhibited by adding paquinimod (39% and 64% reduction, respectively). No effects were found on MMP-9 (Figure 3D).

Discussion

In the current study we show that the S100A9 inhibitor paquinimod reduces pathology in experimental OA that is characterized by high synovial activation. Furthermore, we show that paquinimod can reduce induction of pro-inflammatory and catabolic mediators by S100A9 in human OA synovium.

We showed earlier that paquinimod binds S100A9, but not S100A8, using photoaffinity labelling and surface plasmon resonance (SPR) (10). Furthermore, we found that S100A9-/- mice showed reduction of synovial thickening, osteophyte formation and cartilage damage during CIOA (5). Therefore, the beneficial effects of paquinimod on experimental OA are probably due to a specific inhibition of functional S100A9. We recently showed that the effects of paquinimod on encephalomyelitis run via Ly6Chi monocytes (9), providing another possible mechanism for the observed effects on OA pathology. In recent years, researchers and clinicians have given more focus to the role of inflammation in OA and it is believed that synovial inflammation might be one of the parameters to classify different phenotypes of OA patients (16). We found that the effect of paquinimod was largest in an OA model with high synovial activation (CIOA), compared to low (DMM).

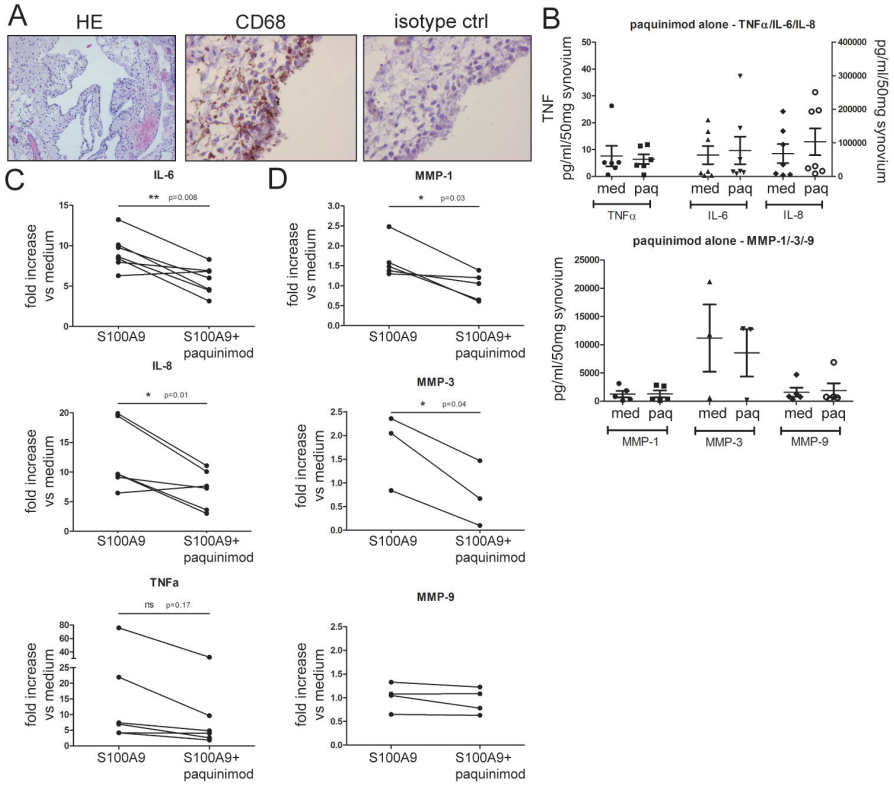

Figure 3: Paquinimod reduces induction of pro-inflammatory and catabolic mediators by S100A9 in human OA synovium

Synovia were collected from end-stage OA patients undergoing arthroplasty, characterized and stimulated for 24 hours with 1µg/ml S100A9 and/or 350 µM paquinimod. **A**. Paraffin-embedded histological sections were stained with hematoxylin/eosin and showed synovial tissue with thickened lining and blood vessels. Staining with specific human CD68 antibodies revealed abundant presence of CD68+ macrophages, mostly in the lining layer. Isotype IgG staining was negative. (Magnification 400x) **B**. Treatment of OA synovia with 350 µM paquinimod did not affect production of pro-inflammatory mediators TNFα, IL-6 and IL-8, nor of catabolic MMP-1, -3 and -9 (IL-1β and MMP-13 were not detectable). Every dot represents one donor. med=medium, paq=paquinimod **C**. S100A9 upregulated release of IL-6, -8 and TNFα by synovial specimen and addition of paquinimod significantly inhibited this upregulation for IL-6 and -8, while a clear trend towards inhibition was observed for TNFα. **D**. S100A9 upregulated release of MMP-1 and -3 by OA synovia and this effect was significantly inhibited by addition of paquinimod. MMP-9 release was not affected by S100A9 with or without paquinimod. In **C** and **D** every line represents one donor. Measurements were done in 7 donors in total, although in some cytokine or MMP levels were below detection level. * = P<0.05, ** = P<0.01 as measured by students t-test.

In translation of this study to the clinic, one could imagine that targeting S100A9 by paquinimod would especially be suitable for treating patients with considerable synovial activation and high S100A8/A9 levels. Levels of S100A8/A9 in serum could be used as marker to assess synovial inflammation, as they have been described as inflammatory markers in various forms of arthritis like rheumatoid arthritis, juvenile idiopathic arthritis and gout (17-19). Recently, we described presence of S100A8/A9 in serum of early symptomatic OA patients correlating with joint damage and osteophyte size (Chapter 3 and (5)). We now show that paquinimod is able to block production of pro-inflammatory and catabolic mediators induced by S100A9 in human OA synovium, in which CD68+ macrophages are present. This suggests that effects of paquinimod ex vivo run via synovial macrophages. We have shown earlier that macrophages are crucial in mediating OA pathology as selective removal of macrophages prior to CIOA effectively reduces synovial activation, fibrosis, osteophyte formation and MMP-induced cartilage damage (20, 21).

Taken together, our study indicates that paquinimod may be a promising compound to targeting joint destruction, inflammation and osteophyte formation in OA patients with high synovial activation and expressing high levels of S100A8/A9.

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References

1. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). Osteoarthritis Cartilage. 2013;21(1):16-21.

2. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis -- results of a 1 year longitudinal arthroscopic study in 422 patients. Osteoarthritis Cartilage. 2005;13(5):361-7.

3. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. Bone. 2012;51(2):249-57.

4. de Lange-Brokaar BJE, Ioan-Facsinay A, van Osch GJVM, Zuurmond AM, Schoones J, Toes REM, et al. Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. Osteoarthritis Cartilage. 2012;20(12):1484-99.

5. van Lent PLEM, Blom AB, Schelbergen RFP, Sloetjes A, Lafeber FPJG, Lems WF, et al. Active involvement of alarmins S100A8 and S100A9 in the regulation of synovial activation and joint destruction during mouse and human osteoarthritis. Arthritis Rheum. 2012;64(5):1466-76.

6. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007;13(9):1042-9.

7. Schelbergen RFP, Blom AB, van den Bosch MHJ, Sloetjes A, Abdollahi-Roodsaz S, Schreurs BW, et al. Alarmins S100A8 and S100A9 elicit a catabolic effect in human osteoarthritic chondrocytes that is dependent on Toll-like receptor 4. Arthritis Rheum. 2012;64(5):1477-87.

8. Bengtsson AA, Sturfelt G, Lood C, Ronnblom L, van Vollenhoven RF, Axelsson B, et al. Pharmacokinetics, tolerability, and preliminary efficacy of paquinimod (ABR-215757), a new quinoline-3-carboxamide derivative: studies in lupus-prone mice and a multicenter, randomized, double-blind, placebo-controlled, repeat-dose, dose-ranging study in patients with systemic lupus erythematosus. Arthritis Rheum. 2012;64(5):1579-88.

9. Helmersson S, Sundstedt A, Deronic A, Leanderson T, Ivars F. Amelioration of experimental autoimmune encephalomyelitis by the quinoline-3-carboxamide paquinimod: reduced priming of proinflammatory effector CD4(+) T cells. Am J Pathol. 2013;182(5):1671-80.

10. Bjork P, Bjork A, Vogl T, Stenstrom M, Liberg D, Olsson A, et al. Identification of human S100A9 as a novel target for treatment of autoimmune disease via binding to quinoline-3-carboxamides. PLoS biology. 2009;7(4):e97.

11. van der Kraan PM, Vitters EL, van Beuningen HM, van de Putte LB, van den Berg WB. Degenerative knee joint lesions in mice after a single intra-articular collagenase injection. A new model of osteoarthritis. J Exp Pathol (Oxford). 1990;71(1):19-31.

12. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. Osteoarthritis Cartilage. 2007;15(9):1061-9.

13. Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, et al. Osteoarthritis cartilage histopathology: grading and staging. Osteoarthritis Cartilage. 2006;14(1):13-29.

14. van der Kraan PM, Vitters EL, van de Putte LB, van den Berg WB. Development of osteoarthritic lesions in mice by "metabolic" and "mechanical" alterations in the knee joints. The American journal of pathology. 1989;135(6):1001-14.

15. Vogl T, Leukert N, Barczyk K, Strupat K, Roth J. Biophysical characterization of S100A8 and S100A9 in the absence and presence of bivalent cations. Biochim Biophys Acta. 2006;1763(11):1298-306.

16. Conaghan PG. Osteoarthritis in 2012: Parallel evolution of OA phenotypes and therapies. Nat Rev Rheumatol. 2013;9(2):68-70.

17. Gerss J, Roth J, Holzinger D, Ruperto N, Wittkowski H, Frosch M, et al. Phagocyte-specific S100 proteins and high-sensitivity C reactive protein as biomarkers for a risk-adapted treatment to maintain remission in juvenile idiopathic arthritis: a comparative study. Ann Rheum Dis. 2012;71(12):1991-7.

18. Choi IY, Gerlag DM, Herenius MJ, Thurlings RM, Wijbrandts CA, Foell D, et al. MRP8/14 serum levels as a strong predictor of response to biological treatments in patients with rheumatoid arthritis. Ann Rheum Dis. 2013.

19. Holzinger D, Nippe N, Vogl T, Marketon K, Mysore V, Weinhage T, et al. Myeloid-related proteins 8 and 14 contribute to monosodium urate monohydrate crystal-induced inflammation in gout. Arthritis & rheumatology. 2014;66(5):1327-39.

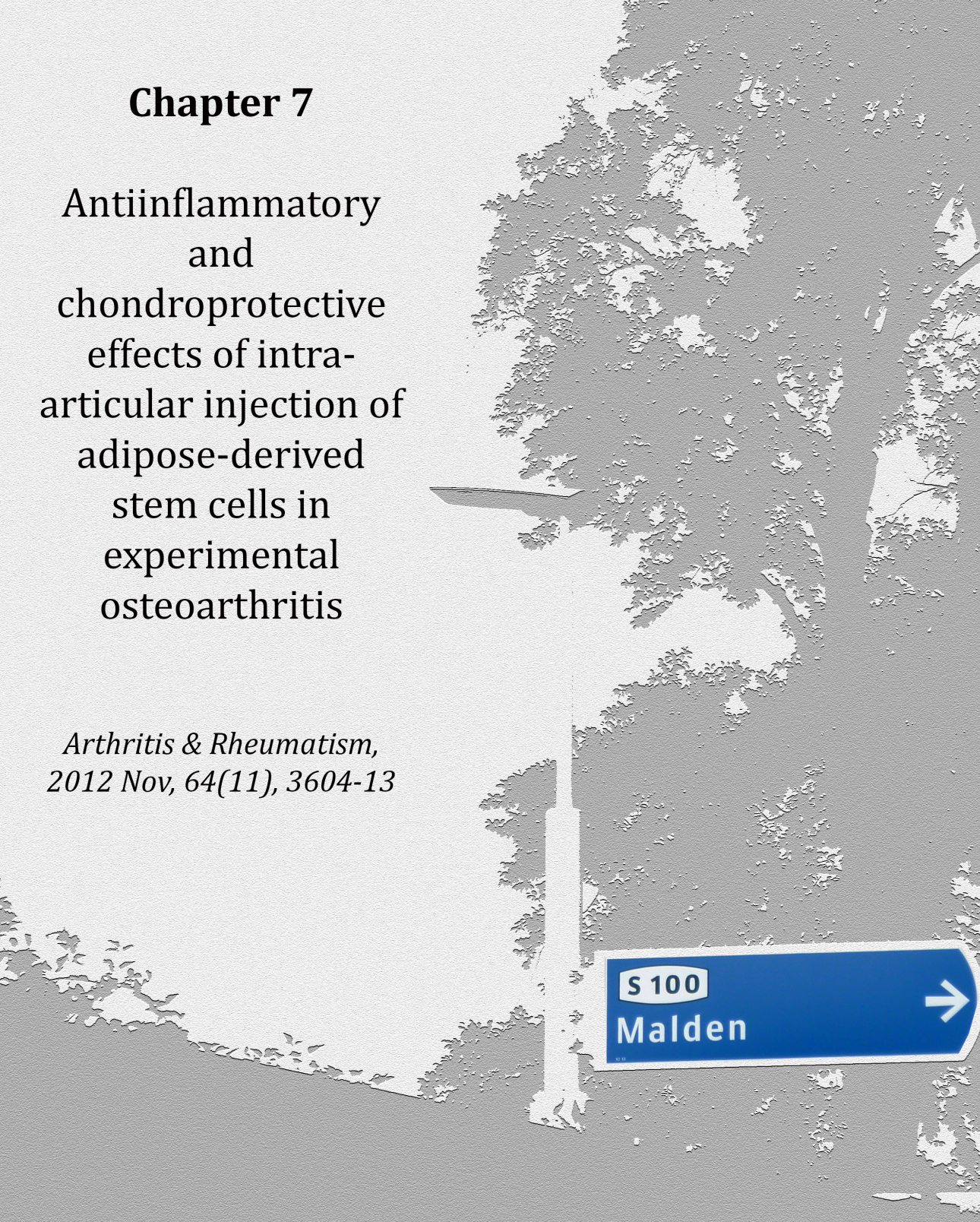
20. Blom AB, van Lent PLEM, Holthuysen AEM, van der Kraan PM, Roth J, van Rooijen N, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. Osteoarthritis Cartilage. 2004;12(8):627-35.

21. Blom AB, van Lent PL, Libregts S, Holthuysen AE, van der Kraan PM, van Rooijen N, et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. Arthritis Rheum. 2007;56(1):147-57.

Chapter 7

Antiinflammatory and chondroprotective effects of intra-articular injection of adipose-derived stem cells in experimental osteoarthritis

Arthritis & Rheumatism, 2012 Nov, 64(11), 3604-13



Chapter 7

Antiinflammatory and Chondroprotective Effects of Intraarticular Injection of Adipose-Derived Stem Cells in Experimental Osteoarthritis

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Arthritis & Rheumatism, 2012 Nov, 64(11), 3604-13

Abstract

Objective: In experimental collagenase-induced osteoarthritis (OA) in the mouse, synovial lining macrophages are crucial in mediating joint destruction. It was recently shown that adipose-derived stem cells (ASCs) express immunosuppressive characteristics. This study was undertaken to explore the effect of intraarticular injection of ASCs on synovial lining thickness and its relation to joint pathology in experimental mouse OA.

Methods: ASCs were isolated from fat surrounding the inguinal lymph nodes and cultured for 2 weeks. Experimental OA was induced by injection of collagenase into the knee joints of C57BL/6 mice. OA phenotypes were measured within 8 weeks after induction. Histologic analysis was performed, and synovial thickening, enthesophyte formation, and cartilage destruction were measured in the knee joint.

Results: ASCs were injected into the knee joints of mice 7 days after the induction of collagenase-induced OA. On day 1, green fluorescent protein–labeled ASCs were attached to the lining layer in close contact with macrophages. Thickening of the synovial lining, formation of enthesophytes associated with medial collateral ligaments, and formation of enthesophytes associated with cruciate ligaments were significantly inhibited on day 42 after ASC treatment, by 31%, 89%, and 44%, respectively. Destruction of cartilage was inhibited on day 14 (65%) and day 42 (35%). In contrast to early treatment, injection of ASCs on day 14 after OA induction showed no significant effect on synovial activation or joint pathology on day 42.

Conclusion: These findings indicate that a single injection of ASCs into the knee joints of mice with early-stage collagenase-induced OA inhibits synovial thickening, formation of enthesophytes associated with ligaments, and cartilage destruction.

Introduction

Although it is generally accepted that osteoarthritis (OA) is a disease of the cartilage, other tissues like subchondral bone and synovium are thought to be involved in mediating joint destruction within this disease. Clinically, patients with OA have a variable degree of synovitis, as was demonstrated by high levels of interleukin (IL)-1β, tumor necrosis factor (TNF)α and complement factors (1, 2). A substantial subpopulation of patients with early symptomatic OA shows an increase in synovial lining layer thickness (3). Synovial biopsies of OA patients show hyperplasia and an increased number of lining cells that mainly consist of CD68+ macrophages. Macrophages within these OA biopsies exhibit an activated phenotype and produce pro-inflammatory cytokines like IL-1β, TNFα, vascular endothelial growth factor (VEGF) and S100-damage-associated molecular patterns (DAMPs) (3, 4), suggesting that these cells may play an important role in OA pathology (5).

Synovial lining macrophages are crucial in joint destruction in experimental collagenase-induced osteoarthritis (CIOA) in mice. In a previous study we found that selective elimination of lining macrophages from the mouse knee joint prior to induction of CIOA, strongly inhibited synovial thickness, development of cartilage destruction and new cartilage/bone, indicating that synovial macrophages mediate joint destruction within this model(6). Synovial macrophages are an important source of both catabolic and anabolic factors which contribute to either cartilage destruction or formation of osteophytes (7).

Mesenchymal stem cells (MSCs) which reside in various tissues of the body have recently been shown to exhibit immunosuppressive characteristics. Although it has generally been accepted that the primary effect of stem cell treatment occurs through tissue specific differentiation (8,9) new data suggest that the therapeutic potential of these cells might be related to their paracrine effect(10,11). Several studies showed that MSCs can modulate the functions of cells of the adaptive immune system, like T and B cells(12). Other studies have shown that these stem cells are also able to induce expression of anti-inflammatory mediators, like IL-10 and IL-12p40, in macrophages(13,14). Multiple factors secreted by MSCs that exert an immunosuppressive effect have been identified, e.g. IL-10, IL-1 receptor

antagonist (IL-1RA), indoleamine 2,3-dioxygenase (IDO), transforming growth factor(TGF)β and prostaglandin E2 (PGE2)(15). Although several contradictory results have been published, there is ample evidence that inflammatory molecules may provide the licensing signal for MSCs to deliver the immunosuppressive signals, including PGE2(10, 16, 17).

Adipose tissues are well known for their key role in energy balance and metabolic disorders. Recently it appeared that adipose tissue hosts multipotent stem cells. These cells named adipose-derived stem cells (ASCs) can easily be purified after digestion of fat and selection by adhesion onto plastic from the very heterogeneous crude stromal fraction. ASCs share numerous properties with bone marrow-derived MSCs(18). In adipose tissue ASCs represent around 5% of nucleated cells in the normal vascular fraction which is much higher than in the adult human bone marrow where MSCs represent only 0.01-0.0001% of the nucleated cells. Both types are able to differentiate towards adipogenic, osteoblastic and chondrogenic phenotype. Moreover they display immunosuppressive properties in vitro as well as in vivo (19). Because of their immunosuppressive phenotype ASCs may be used to decrease local inflammation during rheumatic diseases(20).

In the present study we investigated the effect of local administration of ASCs into the knee joints of mice with experimental induced osteoarthritis in which synovial involvement is evident.

Materials & Methods

Animals

C57BL6 mice were obtained from Janvier (Le Genest St. Isle, France). Animals were between 12 and 14 weeks and received a standard diet and tap water ad libitum. All experiments were approved by the local authority Animal Care and Use Committee and were performed by personnel certified by the Dutch Ministry of WVC.

Induction of experimental osteoarthritis

Experimental CIOA was induced as previously described (21). Briefly, the right knee joint of the mice was injected with 1 unit collagenase type VII from Clostridium histolyticum (Sigma Aldrich, St. Louis, USA) in 6 µl of physiologic saline at day 0 and day 2. Mice were sacrificed at days 14 and 42 after treatment with ASCs. The injected collagenase causes instability of the joints by damaging ligaments and does not directly digest cartilage. This model is characterized by OA-like damage. Clear synovial activation is observed within this model which is related to cartilage destruction(23).

Adipose Stem Cells

ASCs were isolated from adipose tissue surrounding the inguinal lymph nodes. Next, they were cultured for two weeks according to standard procedures in DMEM/F12 (Gibco, New York, USA) supplemented with 1% Penicillin / Streptomycin (Invitrogen, Carlsbad, CA, USA), 0.5% Amphotericin B (Invitrogen, Carlsbad, CA, USA), 16µM Biotin (Sigma Aldrich, St. Louis, USA), 18µM Panthotenic Acid (Sigma Aldrich, St. Louis, USA), 100µM Ascorbic Acid (Sigma Aldrich, St. Louis, USA). After recovery, the cells were tested for 6 different stem cell markers. In accordance with literature, the stem cells stained positive for Sca-1 (86%), CD44 (81%) and CD105 (69%) (BD Bioscience, Breda, the Netherlands; Biolegend, San Diego, CA, USA and eBioscience, San Diego, CA, USA) and were negative for CD11b (0%), cKit (0%) and CD34 (4%) (Biolegend, San Diego, CA, USA; BD Bioscience, Breda, the Netherlands and eBioscience, San Diego,CA,USA). 20.000 cells (derived from second passage) in 6 µL mouse serum (Jackson Immunoresearch, Newmarket, UK) with 4% mouse albumin (Sigma Aldrich, St. Louis, USA) were injected intra-articularly into the knee joint. The number of cells that we used was calculated on basis of the number of cells per kg body weight) that is going to be used in the clinical trial in OA patients (5.106 cells/ joint). Control animals were injected with only mouse serum containing 4% mouse albumin.

Immunodetection of GFP-labeled ASCs

To localize ASCs after intra-articular injection, ASCs were labelled with green fluorescent protein (GFP) prior to injection. After standard culture procedures,

ASCs were transfected with a lentiviral construct containing GFP under a constitutively active PGK promoter. The expression of GFP was confirmed using FACS and fluorescence microscopy. ASCs were injected intra-articularly in mice, 7 days after induction of collagenase-induced OA. 24 Hours after injection, knee joints were isolated and processed for histology. After deparaffinization and rehydration, sections were incubated for 2 hours at room temperature with 10 mM citrate buffer (0.1M citrate acid, 0.1M sodium acid, (pH 8.0)) to unmask antigenic sites. Next, endogenous peroxidase activity was blocked to avoid background staining using 1% H2O2 in methanol. Subsequently, the slides were incubated with a rabbit-anti-GFP antibody (Cell Signaling, Beverly, MA, USA) over night at 4°C. After incubation with a biotinylated goat-anti-rabbit IgG secondary antibody (45 minutes at room temperature) peroxidase labelled avidin-biotin complexes were incubated on the sections using an ABC-kit (Vector Laboratories, Burlingame, CA, USA). Di-amino-benzidine (Sigma Aldrich, St. Louis, USA) was used to localize the peroxidise activity, whereas hematoxylin was used as counterstaining. Finally, the stained sections were mounted in permount (Fisher Scientific, Waltham, MA, USA).

Histological analysis of OA progression

Knee joints were dissected and fixed in 4% formalin. After fixation, the joints were decalcified in 4% formic acid buffered in PBS and processed for histology. Paraffin embedded knee joints were cut in sections of 7μm. Six sections, spaced 140 μm, were mounted on glass slides and stained with safranin O (Saf O) for analysis of cartilage damage and chondrophyte/osteophyte associated ligaments (collateral/cruciate). For scoring of synovial activation, haematoxylin eosin (HE) stained sections were used. Cartilage damage starts with surface fibrillation at day 21 and lesions were observed between day 35 and 42 and was scored using a modified form of the OARSI cartilage OA histopathology grading system (23). Briefly, the depth of the cartilage damage as well as the extent of the damaged surface was scored in a blinded manner at four different locations in the knee joint, i.e. the lateral and medial tibia and femur using an arbitrary score (severity of cartilage destruction 0 to 6 and the extend of damaged cartilage surface 0 to 5). The OA score was defined as a multiplication of these two arbitrary scores. Synovial thickness/activation was scored on HE stained sections using an arbitrary score from 0-3 as previous described (21). Synovial thickening starts at day 7, is maximal at day 14 but is still significant at day 42. Briefly,

scores ranged from no thickening of the lining layer (one cell layer) (0) up to the maximal observed thickening (3) of the synovial lining layer (10-12 cell layers). Saf-O stained sections were used to assess size and number of chondrophyte/ enthesophytes associated with ligaments. Chondrogenesis starts between day 7 and 14 and transformed into bone between day 35 and 42. Size of chondrogenisis was scored in each joint by measurement of three sections, each spaced 140 μm apart. In each section, surface areas of chondrophytes/enthesophytes were measured in cruciate ligaments (CL) and medial collateral ligament (MCL) using an image analysis system (Leica Qwin, Leica Microsystems B.V., Rijswijk, The Netherlands). To assess cartilage formation in the CL, image analysis was used to calculate the surface percentage of the CL positive for proteoglycans. In the MCL the cartilaginous and osseous structures that developed were defined as being chondrophytes or enthesophytes by hand. The mean area per knee joint (three sections) was calculated and expressed in μm^2.

Immunodetection of NITEGE-epitopes

After deparaffinization and rehydration, knee joint sections were treated with citrate buffer and 1% H2O2 in methanol as described above. Thereafter, sections were incubated with anti NITEGE antibodies (Dr. John Mort, Montreal,Canada) in phosphate-buffered saline containing 5% milk powder, 3% fetal calf serum and 2% BSA. Subsequently, sections were incubated with biotinylated goat-anti-rabbit IgG secondary antibody. After incubation with peroxidase labelled avidin-biotin complexes (Vector Laboratories, Burlingame, CA, USA) di-amino-benzidine (DAB) (Sigma Aldrich, St. Louis, USA) was used to localize the peroxidase activity. Sections were counterstained with hematoxylin for 1 min and embedded in Permount (Fisher Scientific, Waltham, MA, USA).

Isolation of murine synovial specimen and preparation of RNA and RT-PCR

At day 14 after ADSC treatment of a day 7 osteoarthritis knee joint, synovial specimen were isolated as described previously (9). In short, joint capsule specimens were isolated on medial and lateral sides of the patella with a biopsy punch. Synovial specimen were snap frozen in liquid nitrogen and stored for RNA isolation. RNA was isolated from synovium using TRIzol reagent according to the manufacturer's protocol (Invitrogen). Five μg of total RNA was reverse transcribed, and cDNA aliquots were subjected to PCR. RT-PCR was normalized

by the transcriptional levels of GAPDH. mRNA levels of various members of cytokines (IL-1β, IL-6, IL-10) were measured in synovial specimen at various time-points after induction of collagenase-induced or DMM osteoarthritis and were quantified using the ABI/PRISM 7000 Sequence Detection System.

Statistical analysis

Statistical differences were calculated with the Mann Whitney U test, using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA). Differences were called significant with P-values < 0.05.

Results

Decrease in synovial activation after ASC treatment

A thickened synovial lining layer comprising activated macrophages is prominent in CIOA and has earlier been shown to be involved in mediating cartilage destruction and osteophyte formation (7). Hyperplasia of the synovial lining layer is maximal at day 7 of CIOA and remains high thereafter.

To test whether ASCs can alter synovial lining thickness, a single injection of ASCs ($2 \bullet 10^4$ cells in 6 μl mouse serum containing 4% MSA) was given into the right knee joint at day 7 after induction of CIOA. Synovial lining thickness was scored after injection of either ASCs or serum only, by two independent observers. Figure 1A shows that lining layer thickness was significantly lower at day 42 (31%) but not at day 14 (9% lower) after ASC treatment when compared to control treated mice (Figure 1A). Photomicrographs show that the synovial lining layer of ASC-treated mice is significantly less thickened comprising lower numbers of macrophages when compared to control-treated mice (Figure 1C versus control B).Interesting at day 14 after ASC treatment, mRNA levels of IL-1β (but not that of IL-6 or IL-10) were significantly lower in the OA synovium when compared to control (Figure 1D).

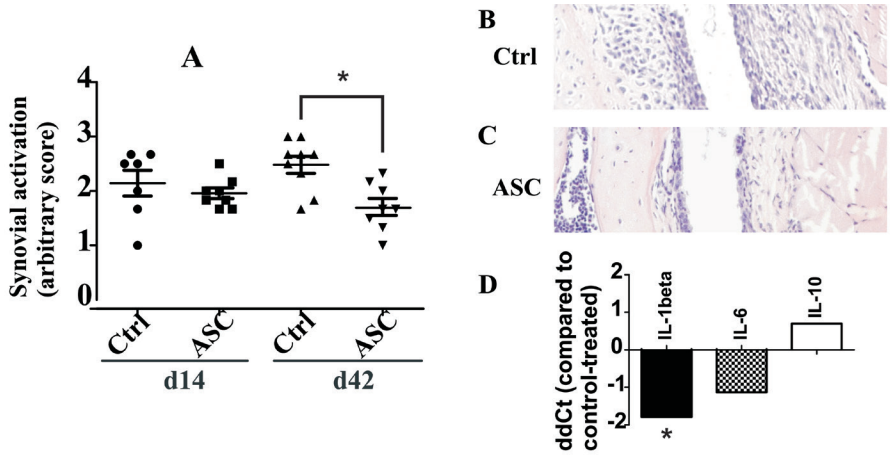

Figure 1. Decrease in synovial thickening in mice treated with adipose-derived stem cells (ASCs).
A, Decreased thickening of the synovium in ASC-treated mice on day 14 and day 42 after treatment. The decrease in thickening versus control mice (31%) reached significance on day 42. Each data point represents a single mouse; horizontal lines and error bars show the mean ± SEM (n = 8–11 mice per group). **B** and **C**, Clear reduction in thickening of the lining layer in an ASC-treated mouse (**C**) compared to a control animal (**B**) on day 42 after treatment. Results are representative of 2 experiments. Original magnification x 250. **D**, Significant reduction in the expression of mRNA for interleukin-1β (IL-1β), but not IL-6 or IL-10, in the osteoarthritic synovium of ASC-treated mice, as compared to controls, on day 14 after treatment. RNA from the synovium of 6 animals per group was used for the analysis of gene expression. Bars show the mean. *=P < 0.05.

Intraarticularly injected ASCs home to the subintimal synovial lining layer in mice

As intra-articular injection of ASCs decreases thickness of the synovial lining, we next analyzed whether ASCs directly interact with the synovium using ASCs labeled with green fluorescent protein (GFP). In vitro, ASCs were transfected with a lentiviral GFP construct and additionally cultured for 24 hours in chamber slides. More than 85% of the ASCs expressed an abundant amount of GFP protein as assessed by flow cytometry (data not shown) and fluorescence microscopy (Figure 2A). Next, GFP-transfected ASCs were injected into a day 7 CIOA knee joint. 24 hours and 5 days thereafter knee joints were isolated and processed for histology. When knee joint sections were immunostained using specific GFP antibodies, GFP-positive cells were clearly visualized in the crucial ligament area and within the subintimal layer of the synovium at 24 hrs (Figure 2B,C). A larger magnification shows that the ASC were lying just below the intima lining layer in

close interaction with synovial macrophages (Figure 2C insert). At 5 days after injection, ASCs were no longer detected in the lining layer (data not shown).

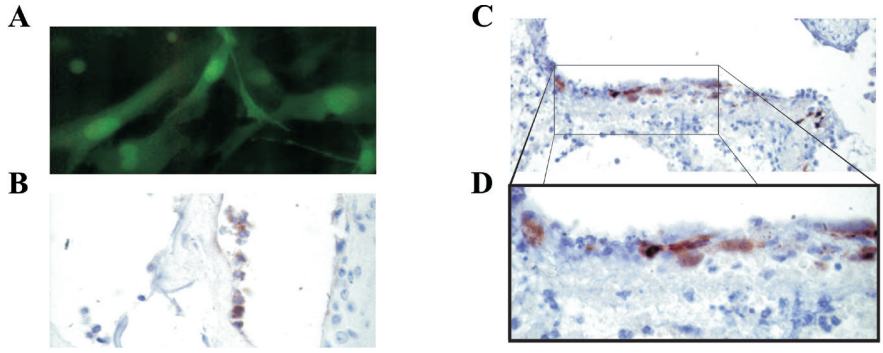

Figure 2. Intraarticularly injected adipose-derived stem cells (ASCs) home to cruciate ligaments and subintimal layers of the synovium in mice.
Green fluorescent protein (GFP)–transfected ASCs were injected into mice on day 7 after the induction of collagenase-induced osteoarthritis. Twenty-four hours after the injection of ASCs, mouse knee joints were isolated and processed, and knee joint sections were stained with an anti-GFP antibody. **A**, Fluorescence microscopy, showing efficient transfection of ASCs with lentiviral GFP. **B**, GFPpositive cells close to the cruciate ligaments. **C** and **D**, Localization of clusters of GFP-positive cells in subintimal layers of the synovium. **D** shows a higher-magnification view of the boxed area in **C**. Original magnification x 1,000 in **A**; x 250 in **B** and **C**; x 400 in **D**.

Prevention of dislocation of the OA knee joint and prevention of new cartilage formation in the collateral and cruciate ligaments in ASC-treated mice

Synovial activation in CIOA strongly contributes to damage of collateral and crucial ligaments leading to destabilization and subsequent dislocation of the knee joint. Investigating total knee joint sections, we observed that induction of CIOA caused dislocation of the knee joint in the majority (75%) of the mice. A single injection of ASC prevented cruciate ligament rupture to only 25% of the mice suggesting that damage to ligaments is protected (data not shown).

Clinically, new formation of cartilage and bone structures within ligaments impairs good functioning of the joint. During experimental OA, early pathological changes comprise new formation of cartilage (chondrophytes) in collateral and cruciate ligaments which subsequently change through ossification into

enthosophytes (21). At day 14 after induction of CIOA, chondrogenesis was already observed in collateral ligaments, particularly at the medial site (Figure 3A). At day 42 after OA induction, these structures had completely transformed into bone (enthesophytes) due to ossification (Figure 3B).

On day 14 after ASC treatment, chondrogenesis (measured as proteoglycan deposition) was mainly observed in the medial collateral ligaments in the majority of the control OA mice. In contrast, much less chondrophyte formation (76% lower) (p=0.08) was observed in the ASC-treated mice (Figure 3E). In line with these observations, significantly smaller enthesophytes (size quantified using image analysis) were measured in the medial lateral collateral ligaments at day 42 after treatment. The surface area decreased by 89% in ASC-treated animals compared to control animals (P < 0.05) (Figures 3C–E).

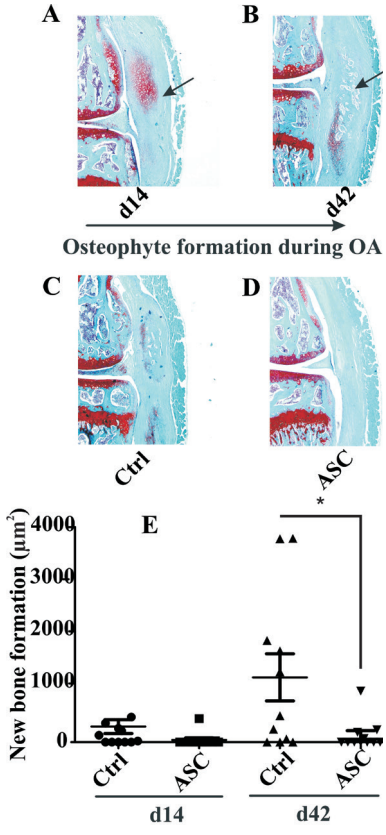

Figure 3. Prevention of chondrogenesis and enthesophyte formation in the medial collateral ligament in mice treated with adipose-derived stem cells (ASCs). On day 14 and day 42 after treatment, chondrogenesis and the size of newly formed bone structures were determined using a Leica QWin image analysis system. **A**, Chondrogenesis (arrow) within the mouse medial collateral ligament on day 14 in an untreated control animal. **B**, Transformation of structures into enthesophytes (arrow) through ossification by day 42 in an untreated control animal. **C** and **D**, Newly formed enthesophytes in a control animal (**C**) and an ASC-treated animal (**D**) on day 42 after treatment. Original magnification x 250 in **A**–**D**. **E**, Decrease in the surface area containing proteoglycans (chondrogenesis) in ASC-treated animals compared to control animals on day 14 and decrease in the surface area of newly formed enthesophytes (by 89%) in ASC-treated animals compared to control animals on day 42. Each data point represents a single mouse; horizontal lines and error bars show the mean ± SEM. Results are representative of 2 experiments (n = 8–11 mice per group). *= P < 0.05.

In addition to the collateral ligaments, the cruciate ligaments of the OA mouse knee joint showed chondrogenesis/enthesophytes, which were measured using image analysis (Figure 4A). Safranin-O staining showed marked presence of proteoglycans at day 42 particularly at the contact sites of the ligaments with the bone. The area that stained positive for proteoglycans was significantly smaller (44%) in ASC-treated mice (Figure 4C) than in controls (Figure 4B). Deposition of proteoglycans was seen in 35% of the ligaments of the ASC-treated mice compared to 63% of the ligaments of the controls ($P < 0.05$) (Figure 4D).

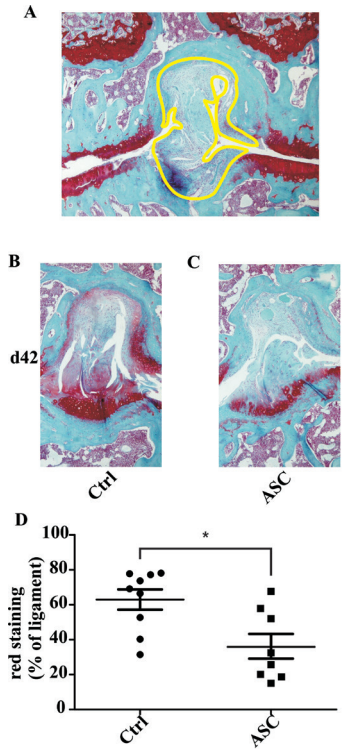

Figure 4. Chondrogenesis in the mouse cruciate ligaments during experimental osteoarthritis.
A, Chondrogenesis in the mouse cruciate ligaments delineated as shown, quantified using a Leica QWin image analysis system. **B** and **C**, Proteoglycan deposition in a control animal (**B**) and an adipose-derived stem cell (ASC)–treated animal (**C**) on day 42 after treatment. Original magnification x 250 in **A**–**C**. **D**, Significant decrease in proteoglycan deposition in ASC-treated animals compared to controls on day 42 after treatment. Each data point represents a single mouse; horizontal lines and error bars show the mean ± SEM (n = 8–9 mice per group). *=P < 0.05.

Synovial activation strongly upregulates enzymes like ADAMTs 4 and 5, members of the aggrecanase family, which are major players involved in new formation and remodelling of cartilage. These enzymes cleave proteoglycans at a specific site thereby leaving neoepitopes (ending at the aminoacid sequence NITEGE) within the cartilage matrix which can be detected using specific antibodies and immunolocalisation. A strong staining of NITEGE neoepitopes was found within the cartilage containing areas of the cruciate ligaments of the control OA

knee joint. A single injection of ASCs significantly inhibited NITEGE neoepitope expression, suggesting that aggrecanase activity is suppressed by ASC treatment (Figure 5). In vitro, ASCs expressed high levels of mRNA for tissue inhibitors of metalloproteinases (TIMPs), particularly TIMPs 1 and 3, when compared to macrophages, suggesting that they may be involved in inhibiting enzyme activity (data not shown).

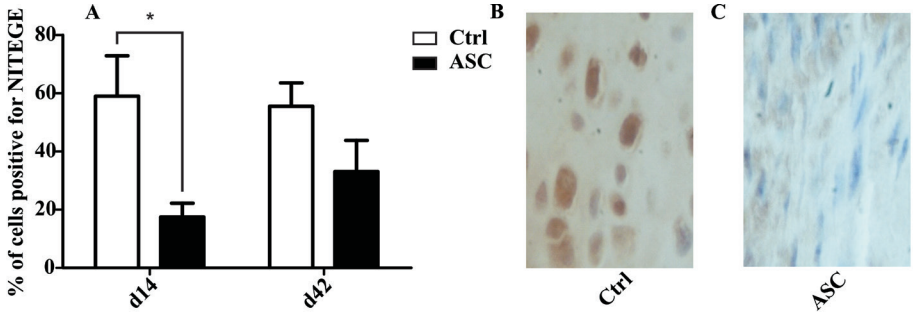

Figure 5. Decreased NITEGE expression in the cruciate ligaments of the knee joints of mice with collagenase-induced osteoarthritis treated with adipose-derived stem cells (ASCs).
The neoepitope NITEGE is expressed upon ADAMTS activity during chondrogenesis/bone formation. The percentage of NITEGE staining regions in the mouse cruciate ligament was determined. **A**, Reduction of 69% and 35% on days 14 and 42, respectively, in NITEGE staining in mice treated with ASC compared to controls. Bars show the mean ± SEM (n = 8 mice per group). *= P < 0.05. **B** and **C**, Hypertrophic, chondrocyte-like phenotype of cells in the cruciate ligaments of a control animal (**B**) compared to an ASC-treated animal (**C**). Original magnification x 250.

Significant inhibition of cartilage destruction in mice with collagenase-induced OA after intraarticular injection of ASCs into the knee joint

Synovial activation promotes destruction of cartilage during CIOA through combined action of catabolic factors and biomechanical ligamen instability induced by anabolic mediators. At day 42 of CIOA , a prominent cartilage destruction was observed in all four layers of the knee joint. A single injection of ASC into the day 7 OA knee joint inhibited cartilage damage already at day 14. In particular, the cartilage damage in the lateral femur was protected (65% lower in ASC-treated mice when compared to control mice; p< 0.05) (Figure 6A). On day 42 after treatment the mean of the OA scores, indicating cartilage damage, for the 4 different locations within the knee joint was still significantly decreased (by 35%) in ASC-treated mice compared to controls (P < 0.05) (Figures 6B–D).

Lack of a significant effect of a single injection of ASCs at a later time point (day 14) on synovial thickness and joint destruction in mice with collagenase-induced OA

Next, we studied whether ASC treatment also affects synovial thickness and joint destruction when given at day 14 after induction of CIOA. At that time-point ligaments are already severely damaged in contrast to day 7 OA. At day 42 after a single injection of ASC into the day 14 OA knee joint, synovial thickness (Figure 6E), enthesophyte formation (Figure 6F) and cartilage destruction (Figure 6G) although somewhat lower, were not significantly reduced when compared to controls.

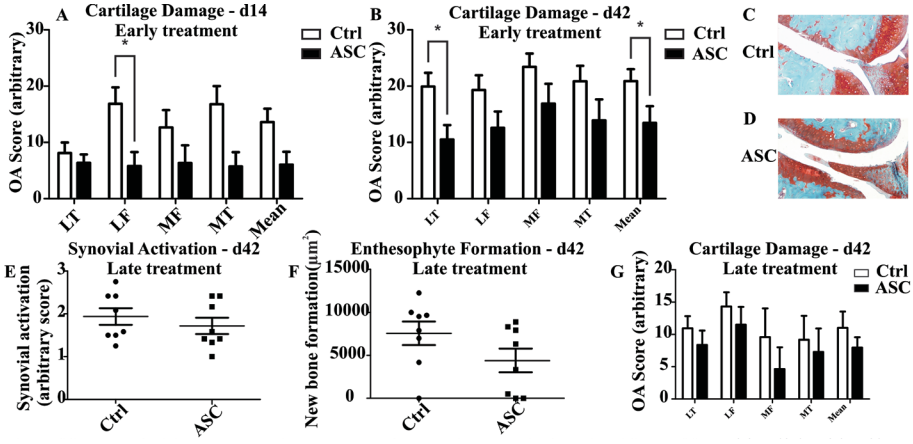

Figure 6. Inhibition of the development of cartilage destruction in mice treated with adipose-derived stem cells (ASCs) soon after the induction of collagenase-induced osteoarthritis (OA).
ASCs were injected into the mouse knee joints on either day 7 or day 14 after the induction of collagenase-induced OA. Cartilage destruction was scored at 4 different locations in the knee joint, i.e., the lateral tibia (LT), lateral femur (LF), medial femur (MF), and medial tibia (MT). The mean OA score was calculated using the scores for the 4 individual locations. **A** and **B**, Reduction in cartilage damage scores on day 14 after treatment (**A**) and day 42 after treatment (**B**) in mice treated with ASCs 7 days after the induction of OA (early treatment) compared to control mice. The difference was significant for the lateral femur score on day 14 and for the lateral tibia score and mean score on day 42. Bars show the mean ± SEM (n = 8 mice per group). *=P < 0.05. **C** and **D**, Cartilage damage on day 42 after treatment in a control mouse (**C**) and a mouse treated with ASCs 7 days after the induction of OA (**D**). Original magnification x 250. **E**–**G**, Lack of a significant effect on synovial thickness (**E**), enthesophyte formation (**F**), or cartilage damage (**G**) on day 42 after treatment in mice treated with ASCs 14 days after the induction of OA (late treatment). In **E** and **F**, each data point represents a single mouse; horizontal lines and error bars show the mean ± SEM. In **G**, bars show the mean ± SEM (n = 8 mice per group).

Discussion

In the present study we find that a single injection of ASC into the knee joint given at an early time-point (day 7) after induction of CIOA, protects against synovial thickening, enthesophyte formation and cartilage destruction.

Stem cells are used in cellular therapy for a broad spectrum of diseases. Although it is generally believed that the primary therapeutic effect of stem cells occurs through tissue differentiation (8,9), they may also act in a paracrine manner by the production of soluble anti-inflammatory factors. Previous studies in our lab have shown that thickening of the synovial lining layer is a characteristic feature of CIOA. This layer comprises mainly macrophages exhibiting an activated phenotype. Increased thickness of the lining starts at day 3, reaches its maximum at day 7 and persisted up to day 42 (24). We showed earlier that selective elimination of the lining macrophages prior to induction of CIOA strongly inhibited synovial thickness but also osteophyte formation and cartilage destruction (6, 24).

Synovial macrophages are important producers of chemokines that attract monocytes which subsequently differentiate into macrophages or induce proliferation of fibroblasts within the lining layer thereby promoting its thickness. During OA, the cartilage layer is damaged and releases cartilage fragments. SLRPs, small proteolycans like biglycan and decorin have been shown to stimulate macrophages via TLR4 thereby releasing pro-inflammatory cytokines IL-1 (25), TNFα or S100-DAMPs(S100A8/A9) (26) which may further stimulate the activation status of the lining layer.

Injection of ASCs at day 7 after onset of CIOA inhibited synovial thickness in the OA knee joint at day 42. Localisation of GFP-labelled ASC in the OA knee joint, showed that 24 hours after injection, ASCs were detected within the synovium just below the lining layer. A close interaction between ASC and macrophages within the intimal layer was observed. At day 5 after injection virtually no GFP-labeled cells could be detected anymore (data not shown) suggesting that the effects on the synovium are induced within the first days after injection initiating a prolonged long lasting suppressive effect.

Chondrogenesis and subsequent formation of enthesophytes within collateral/ cruciate ligaments within this model is mediated by synovial macrophages (6, 24) and hampers a good functioning of the joint which may further enhance joint destruction. Macrophage derived factors involved in chondrogenesis include growth factors like TGFβ, BMP-2 and -4(6). Production of these growth factors within the synovium was strongly diminished after macrophages were selectively depleted from the lining layer(24). During OA, dysregulated chondrogenesis is observed along the margins of the articular cartilage or within the ligaments and most likely differentiate from chondrogenic precursor cells that reside within periosteum or ligament. Stem cell treatment may suppress production of growth factors by synovial macrophages thereby preventing proliferation of chondrogenic precursor cells.

Damage of the cruciate ligaments was strongly correlated to dislocation of the knee joint. In the cruciate ligament area, macrophages are also abundantly present and prolonged activation of these cells may further stimulate ligament destruction. Recent studies showed that enzymes like ADAMTs are involved in degeneration of ligament structures(27) and are crucial for destruction of cartilage in experimental OA(28). These enzymes cleave aggrecan thereby inducing neoepitopes (NITEGE) which can be visualized with immunodetection(27). Stem cell treatment strongly inhibited expression of NITEGE neoepitopes suggesting that ADAMTs activity is suppressed.

Cartilage destruction is a characteristic feature of OA which was also inhibited by local ASC treatment. ASC may suppress macrophage activity and thereby the production of catabolic mediators like IL-1β and TNFα. Recently we described that “alarmins” S100A8/S100A9, major products of macrophages, are key players in mediating cartilage destruction in CIOA(28, 29). These proteins stimulate MMP production in macrophages(30) and chondrocytes(29) via TLR4 signalling. IL-1β is expressed only during the first phase whereas S100 proteins are released throughout the CIOA course and may explain why S100 proteins are dominant within this model. Whether ASC treatment has effect on IL-1β, TNFα and S100A8/A9 levels in the blood and synovium washouts is momentarily under investigation.

When ASCs were administered intra-articularly at day 14 after onset of CIOA no significant protection was found on joint pathology. One explanation may be that

collateral/cruciate ligaments are already irreversibly damaged at that time-point preventing successful recovery. Mediators like IL-1β and MMPs have predictive value for ligament injury in animal models (31). Stem cells have been shown to produce soluble inhibiting factors like IL-1RA (32) and TIMPs (33) which may partly block induction of the IL-1/MMP mediated ligament destruction (31). However an earlier study has shown that late delivery of autologous MSCs to caprine joints subjected to meniscectomy and complete resection of the anterior cruciate ligament still resulted in regeneration of meniscal tissue and significant chondroprotection (34). In our study we only injected a low amount of ASC in the knee joint which may be sufficient for inhibiting induction of ligament damage but may be too low for repair of already damaged ligaments. Damaged ligaments can bind MSCs thereby providing the basis for a biological repair of this ligament (35).

A further explanation that deposition of ASCs may not be effective when given at day 14 after CIOA may be the minor release of cytokines by the synovium at that late time-point. Pro-inflammatory cytokines, like IFNγ, IL-6 and TNF-α, have shown to enhance the immunosuppressive potential in ASCs (13, 16, 17, 36). The release of pro-inflammatory factors is highest within the synovium during the first seven days after onset of CIOA (28) and may drive the anti-inflammatory potential of ASCs. In reaction to pro-inflammatory stimuli, ASC increase intracellular enzymes Indolamine 2,3, dioxigenase (IDO), heme-oxygenase-1 (HO-1) and COX-2 leading to enhanced release of CO, biliverdin and PGE2 which are able to suppress activated macrophages thereby preventing OA pathology (13, 37-38). This mechanism may further explain why stem cell treatment is so effective in inflammatory diseases like arthritis (16) and ischemic heart disease (39). Stimulation of ASCs with pro-inflammatory cytokines prior to delivery to the OA joint may further enhance the efficacy of ASC treatment.

In the present paper we show that ASCs when given at an early phase of experimental OA inhibit synovial lining thickness and protect joint destruction by both anabolic and catabolic mediators. ASCs may therefore be a potent and safe tool for therapeutic approach of a disease from which up till now no cure is available.

Acknowledgements

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References

1. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol. 2011;7(1):33-42.

2. Wang Q, Rozelle AL, Lepus CM, Scanzello CR, Song JJ, Larsen DM, et al. Identification of a central role for complement in osteoarthritis. Nat Med. 2011;17(12):1674-9.

3. Haywood L, McWilliams DF, Pearson CI, Gill SE, Ganesan A, Wilson D, et al. Inflammation and angiogenesis in osteoarthritis. Arthritis Rheum. 2003;48(8):2173-7.

4. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis. 2005;64(9):1263-7.

5. Bondeson J, Blom AB, Wainwright S, Hughes C, Caterson B, van den Berg WB. The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis. Arthritis Rheum. 2010;62(3):647-57.

6. van Lent PL, Blom AB, van der Kraan P, Holthuysen AE, Vitters E, van Rooijen N, et al. Crucial role of synovial lining macrophages in the promotion of transforming growth factor beta-mediated osteophyte formation. Arthritis Rheum. 2004;50(1):103-11.

7. Blom AB, van Lent PL, Libregts S, Holthuysen AE, van der Kraan PM, van Rooijen N, et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. Arthritis Rheum. 2007;56(1):147-57.

8. O'Sullivan J, D'Arcy S, Barry FP, Murphy JM, Coleman CM.Mesenchymal chondroprogenitor cell origin and therapeutic potential. Stem Cell Res Ther. 2011 18;2(1):8.

9. Coleman CM, Curtin C, Barry FP, O'Flatharta C, Murphy JM. Mesenchymal stem cells and osteoarthritis: remedy or accomplice? Hum Gene Ther. 2010;21(10):1239-50.

10. Maumus M, Guérit D, Toupet K, Jorgensen C, Noël D. Mesenchymal stem cell-ased therapies in regenerative medicine: applications in rheumatology. Stem Cell Res Ther. 2011 Mar 18;2(2):14.

11. Coleman, CM, Curtin C, Barry FP, O'Flatharta C, Murphy JM. Mesenchymal stem cells and osteoarthritis: remedy or accomplice? Hum Gene Ther, 2010; 21(10): 1239-50.

12. Abumaree M, Al Jumah M, Pace RA, Kalionis B. Immunosuppressive Properties of Mesenchymal Stem Cells. Stem Cell Rev. 2011.

13. Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med. 2009;15(1):42-9.

14. Maggini J, Mirkin G, Bognanni I, Holmberg J, Piazzon IM, Nepomnaschy I, et al. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. PLoS One. 2010;5(2):e9252.

15. Pittenger M. Sleuthing the source of regeneration by MSCs. Cell Stem Cell. 2009;5(1):8-10.

16. Bouffi C, Bony C, Courties G, Jorgensen C, Noel D. IL-6-dependent PGE2 secretion by mesenchymal stem cells inhibits local inflammation in experimental arthritis. PLoS One. 2010;5(12):e14247.

17. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur J Immunol. 2008;38(6):1745-55.

18. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, et al. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br J Haematol. 2005;129(1):118-29.

19. De Toni F, Poglio S, Youcef AB, Cousin B, Pflumio F, Bourin P, et al. Human adipose-derived stromal cells efficiently support hematopoiesis in vitro and in vivo: a key step for therapeutic studies. Stem Cells Dev. 2011;20(12):2127-38.

20. Djouad F, Bouffi C, Ghannam S, Noel D, Jorgensen C. Mesenchymal stem cells: innovative therapeutic tools for rheumatic diseases. Nat Rev Rheumatol. 2009;5(7):392-9.

21. van der Kraan PM, Vitters EL, van Beuningen HM, van de Putte LB, van den Berg WB. Degenerative knee joint lesions in mice after a single intra-articular collagenase injection. A new model of osteoarthritis. J Exp Pathol (Oxford). 1990;71(1):19-31.

22. Glasson SS, Chambers MG, Van Den Berg WB, Little CB. The OARSI histopathology initiative- recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis Cartilage. 2010;18 Suppl 3:S17-23.

23. Blom AB, van Lent PL, Holthuysen AE, van der Kraan PM, Roth J, van Rooijen N, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. Osteoarthritis Cartilage. 2004;12(8):627-35.

24. Daheshia M, Yao JQ. The interleukin 1beta pathway in the pathogenesis of osteoarthritis. J Rheumatol. 2008;35(12):2306-12.

25. van Lent PL, Grevers L, Blom AB, Sloetjes A, Mort JS, Vogl T, et al. Myeloid-related proteins S100A8/S100A9 regulate joint inflammation and cartilage destruction during antigen-induced arthritis. Ann Rheum Dis. 2008;67(12):1750-8.

26. Plaas A, Sandy JD, Liu H, Diaz MA, Schenkman D, Magnus RP, et al. Biochemical identification and immunolocalizaton of aggrecan, ADAMTS5 and inter-alpha-trypsin-inhibitor in equine degenerative suspensory ligament desmitis. J Orthop Res. 2011;29(6):900-6.

27. Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma HL, et al. Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature. 2005;434(7033):644-8.

28. van Lent P, Blom A, Schelbergen R, Sloetjes A, Lafeber F, Lems W, et al. Active involvement of "alarmins" S100A8 and S100A9 in regulation of synovial activation and joint destruction during mouse and human osteoarthritis. Arthritis Rheum. 2011; doi: 10.1002/art.34315.

29. Schelbergen, R.F., et al., Alarmins S100A8 and S100A9 elicit a catabolic effect in human osteoarthritic chondrocytes that is dependent on toll-like receptor 4. Arthritis Rheum, 2011; doi: 10.1002/art.33495.

30 Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, Nacken W,Foell D, van der Poll T, Sorg C, Roth J. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007 Sep;13(9):1042-9.

31 Heard BJ, Achari Y, Chung M, Shrive NG, Frank CB. Early joint tissue changes are highly correlated with a set of inflammatory and degradative synovial biomarkers after ACL autograft and its sham surgery in an ovine model. J Orthop Res.2011; 29(8):1185-92.

32 Ortiz LA, Dutreil M, Fattman C, Pandey AC, Torres G, Go K, Phinney DG. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. Proc Natl Acad Sci U S A. 2007 ;104(26):11002-7.

33 Tondreau T, Meuleman N, Stamatopoulos B, De Bruyn C, Delforge A, Dejeneffe M,Martiat P, Bron D, Lagneaux L. In vitro study of matrix metalloproteinase/tissue inhibitor of metalloproteinase production by mesenchymal stromal cells in response to inflammatory

cytokines: the role of their migration in injured tissues. Cytotherapy. 2009;11(5):559-69.

34 Murphy JM, Fink DJ, Hunziker EB, Barry FP. Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum. 2003;48(12):3464-74.

35 Steinert AF, Kunz M, Prager P, Barthel T, Jakob F, Nöth U, Murray MM, Evans CH, Porter RM. Mesenchymal stem cell characteristics of human anterior cruciate ligament outgrowth cells. Tissue Eng Part A. 2011;17(9-10):1375-88.

36. Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Pescatori M, Stubbs AP, et al. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clin Exp Immunol. 2010;162(3):474-86.

37. Mitsui H, Aoyama T, Furu M, Ito K, Jin Y, Maruyama T, et al. Prostaglandin E2 receptor type 2-selective agonist prevents the degeneration of articular cartilage in rabbit knees with traumatic instability. Arthritis Res Ther. 2011;13(5):R146.

38 Mougiakakos D, Jitschin R, Johansson CC, Okita R, Kiessling R, Le Blanc K. The impact of inflammatory licensing on heme oxygenase-1-mediated induction of regulatory T cells by human mesenchymal stem cells. Blood. 2011;117(18):4826-35.

39. Herrmann JL, Abarbanell AM, Wang Y, Weil BR, Poynter JA, Manukyan MC, et al. Transforming Growth Factor-alpha Enhances Stem Cell-Mediated Postischemic Myocardial Protection. Ann Thorac Surg. 2011;92(5):1719-25.

Chapter 8

Treatment efficacy of adipose-derived stem cells in experimental osteoarthritis is driven by high synovial activation and reflected by S100A8/A9 serum levels

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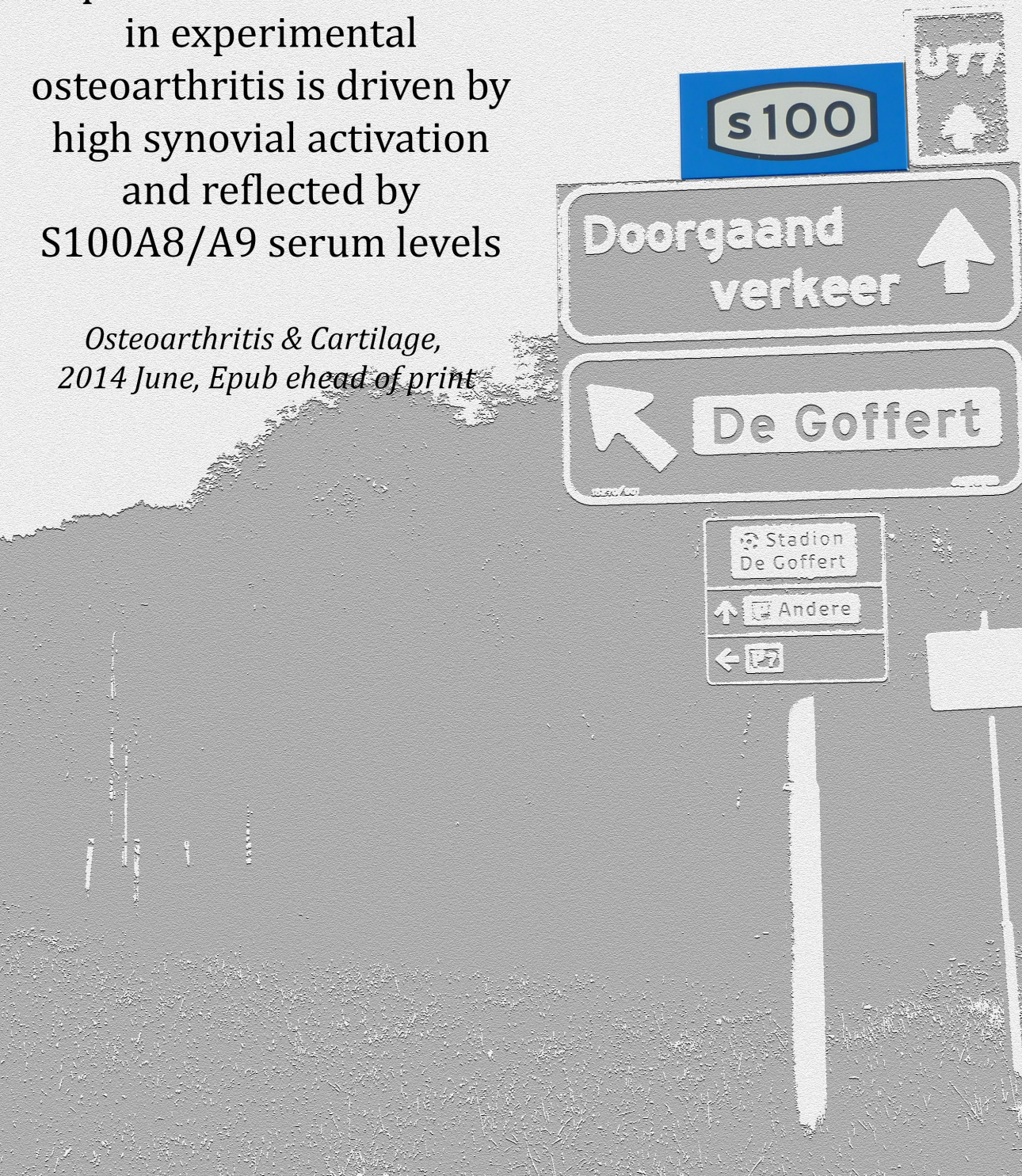

Chapter 8

Treatment efficacy of adipose-derived stem cells in experimental osteoarthritis is driven by high synovial activation and reflected by S100A8/A9 serum levels.

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Abstract

Objective: Synovitis is evident in a substantial subpopulation of patients with osteoarthritis (OA) and is associated with development of pathophysiology. Recently we have shown that adipose-derived stem cells (ASC) inhibit joint destruction in collagenase-induced experimental OA (CIOA). In the current study we explored the role of synovitis and alarmins S100A8/A9 in the immunomodulatory capacity of ASCs in experimental OA.

Method: CIOA, characterized by synovitis, and surgical DMM (destabilization of medial meniscus) OA were treated locally with ASCs. Synovial activation, cartilage damage and osteophyte size were measured on histological sections. Cytokines in synovial washouts and serum were determined using Luminex or enzyme-linked immunosorbent assay (S100A8/A9), mRNA levels with reverse-transcriptase (RT)-qPCR.

Results: Local administration of ASCs at various time-points (days 7 or 14) after DMM induction had no effect on OA pathology. At day 7 of CIOA, already 6 hours after ASC injection mRNA expression of pro-inflammatory mediators S100A8/A9, interleukin-1beta (IL-1β) and KC was down-regulated in the synovium. IL-1β protein, although low, was down-regulated by ASC-treatment of CIOA. S100A8/A9 protein levels were very high at 6 and 48 hours and were decreased by ASC-treatment. The protective action of ASC treatment in CIOA was only found when high synovial inflammation was present at the time of deposition which was reflected by high serum S100A8/A9 levels. Finally, successful treatment resulted in significantly lower levels of serum S100A8/A9.

Conclusion: Our study indicates that synovial activation rapidly drives anti-inflammatory and protective effects of intra-articularly deposited ASCs in experimental OA which is reflected by decreased S100A8/A9 levels.

Introduction

Osteoarthritis (OA) is primarily a disease of the cartilage. However, synovitis is thought to contribute to aggravation of joint pathology as well (1, 2). Up to 50% of OA patients have synovitis, which was demonstrated by magnetic resonance imaging, ultrasonography and arthroscopy (3-5). Histologically, inflammation in the synovial membrane is characterized by high levels of CD68+ macrophages expressing an activated phenotype and presence of pro-inflammatory cytokines like IL-1β and TNFα (3, 6, 7).

Synovial lining macrophages contribute to joint destruction in murine collagenase-induced osteoarthritis (CIOA). When lining macrophages were selectively depleted from a mouse knee joint prior to induction of CIOA, a strongly diminished thickening of the synovium was observed which coincided with inhibition of cartilage degradation and formation of cartilage/bone within ligaments (8). Synovial macrophages produce both catabolic and anabolic factors that contribute to degradation and new formation of cartilage (9). Major catabolic factors produced by activated macrophages are alarmins or damage associated molecular patterns (DAMPs) S100A8 and S100A9 (10). These are found in large amounts in the serum and synovium of OA patients and we recently showed that S100A8/A9 predict development of cartilage destruction in the Dutch CHECK cohort of early symptomatic OA patients and have catabolic effects on OA chondrocytes via TLR-4 (11, 12).

Mesenchymal stem cells (MSCs) have been shown to exhibit immunosuppressive characteristics (13, 14), next to their effect on tissue specific differentiation (15). MSCs can secrete multiple immunosuppressive factors, like IL-10, IL-1 Receptor Antagonist (IL-1RA), Indoleamine 2,3-DiOxygenase (IDO), TGFβ and ProstaGlandin E2 (PGE2) (16) and they can induce anti-inflammatory effects in macrophages (17, 18). Moreover, evidence is gathering that MSCs need an inflammatory milieu to deliver immunosuppressive signals (13, 19, 20). Next to bone marrow, adipose tissue also hosts multipotent stem cells. These adipose-derived stem cells (ASCs) share multiple properties with bone marrow-derived MSCs (21), but are more accessible and abundant (5% vs 0.0001-0.001% of nucleated cells). Both types have adipogenic, osteogenic and chondrogenic

differentiation potential and display immunosuppressive properties in vitro as well as in vivo (22).

Recently, we described that local administration of ASCs in the knee joint in early stage CIOA inhibited synovial activation and largely protected against new cartilage/bone formation within collateral and cruciate ligaments (23). Furthermore, cartilage damage was reduced in various layers of the knee joint. We localized GFP-labeled ASCs within the synovial lining layer in close contact to synovial macrophages (23). The immunosuppressive capacity of the ASCs may thereby be stimulated by the pro-inflammatory cytokines released by macrophages during the early phase of CIOA.

In the present study we investigated whether the suppressive effect of ASCs on experimental induced osteoarthritis is driven by synovitis. Furthermore, we explored the role of S100A8/A9, major products during synovitis, in this anti-inflammatory effect of ASCs.

Materials and Methods

Animals

A total of 120 C57BL6J mice were obtained from Janvier. Animals were male and between 12 and 14 weeks old (average weight 24.5g SD 0.3), housed in filter-top cages with up to 10 animals per cage and received a standard diet and tap water ad libitum.

Induction of experimental osteoarthritis

We used two different models of experimental OA, CIOA and DMM, because they differ in synovial inflammation. Experimental CIOA (which has relatively high synovial inflammation) was induced as previously described (24). Briefly, right knee joints of mice were injected with 1U collagenase type VII from Clostridium histolyticum (Sigma-Aldrich) at day 0 and day 2, causing disruption of the ligaments and local instability of the knee joint. To achieve a CIOA with less synovial activation, only one time 1U collagenase was injected. Experimental

DMM OA (destabilized medial meniscus, a model where synovial activation is scant) was induced by transection of the medial anterior meniscotibial ligament (25). For the DMM-study 8 mice/group were used on day 7 and 10 mice/group for day 14. For the effects of ASCs on synovium in CIOA, 6 mice/group were used on day 2, 14 and 42, 8 mice/group after 6 hours. For the comparison between high and low synovial activation in CIOA, 8 mice/group were used.

Adipose Stem Cells

ASCs were isolated from adipose tissue surrounding the inguinal lymph nodes of mice and cultured for two weeks according to standard procedures in DMEM/F12 (Gibco) supplemented with 1% penicillin/streptomycin (Invitrogen), 0.5% amphotericin B (Invitrogen), 16μM biotin (Sigma-Aldrich), 18μM panthotenic acid (Sigma-Aldrich), 100μM ascorbic acid (Sigma-Aldrich). ASCs stained positive for Sca-1, CD44 and CD105 (BD Bioscience; Biolegend and eBioscience) and negative for CD11b, cKit and CD34 (Biolegend; BD Bioscience and eBioscience). 20.000 cells in 6 μL mouse serum (Jackson Immunoresearch) with 4% mouse albumin (Sigma-Aldrich) were injected intra-articularly into the knee joint. Control animals were injected with only mouse serum containing 4% mouse albumin.

Isolation of murine synovial specimens

At various time points (6 hours, 2, 14 and 42 days) after induction of OA, synovial specimens were isolated as described previously (11). Briefly, joint capsule specimens were isolated on the medial and lateral sides of the patella with a biopsy punch. Synovial specimens were snapfrozen in liquid nitrogen and stored for RNA isolation.

In vitro cross-talk between adipose stem cells and activated macrophages

ASCs (passage 2) were stimulated for 24 hours with 10 ng/ml IFNγ and IL-1β and 1 μg/ml recombinant murine S100A8. Bone marrow cells were harvested from C57Bl6J mice and 1x106 were differentiated into macrophages with M-CSF for 6 days and activated with 10 ng/ml LPS for 24 hours. Consequently, these macrophages were incubated for 24 hours with IFNγ- and IL-1β- stimulated ASC supernatant. Effect on gene expression of suppressive factors in both ASCs and

macrophages was investigated using RT-qPCR.

Preparation of RNA and reverse transcription–qPCR

RNA from murine synovium was isolated by first disrupting synovial tissue with the MagNA Lyser (Roche) 5 times for 20 seconds and then isolating the RNA using the RNeasy-kit (Qiagen) according to the manufacturers protocol. RNA from cultured ASCs and macrophages was isolated using TRI-reagent (Sigma-Aldrich). RNA was reverse transcribed to cDNA and qPCR was performed with specific primers and the SYBR Green Master mix in the ABI Prism 7000 Sequence Detection System (Applied Biosystems/Life Technologies). Expression levels were normalized to GAPDH. Primer sequences were designed on exon-exon transition of murine genes and can be found in Table 1.

Histological analysis of OA progression

Knee joints were dissected and fixed in 4% formalin. After fixation, joints were decalcified in 4% formic acid buffered in PBS and processed for histology. Paraffin embedded knee joints were cut in sections of 7µm, stained with Safranin O (Saf-O) and counterstained with Fast Green for analysis of cartilage damage and chondrophyte/osteophyte associated ligaments (collateral/cruciate). Cartilage damage was scored blinded using a modified form of the Pritzker OARSI OA score, which takes into account the grading and staging components (26). Five sections for each specimen were evaluated by two blinded investigators. Minimal score is 0, indicating no cartilage pathology whatsoever, whereas 30 is the maximum score, indicating highest grade (6) and stage (5). For scoring of synovial activation, Haematoxylin Eosin (HE) staining was used. Synovial thickening/activation was scored using an arbitrary score from 0-3 as previous described (27). Saf-O stained sections were used to measure the size of chondrophytes/osteophytes using an image analysis system (Leica Application Suite, Leica).

Protein measurement of S100A8/A9 and cytokines in murine synovial washouts and serum

S100A8/A9 concentrations were determined in serum or synovial washouts of murine knee joints by a sandwich enzyme-linked immunosorbent assay (ELISA) specifically for murine S100A8/A9 as described previously (28). Cytokines (IL-

1β, IL-6, IL-10, KC, IFNγ) were measured in murine synovial washouts using Luminex multianalyte technology on the Bio-Plex system, in combination with multiplex cytokine kits (Milliplex from Millipore

Measurement of TGFβ-activity

TGFβ-activity was determined by adding 1:5 dilution of synovial washouts overnight to 3T3 fibroblasts transduced with adenoviral CAGA-luciferase (CAGA-luc) with a multiplicity of infection (MOI) of 10 (10 plaque-forming units) per cell, after which luminescence was measured. The plasmid was kindly provided by Dr. Ten Dijke (Department of Molecular Cell Biology, Leiden University Medical Center, The Netherlands). The CAGA-boxes in the vector are transcribed by Smad3/4 through active TGFβ, resulting in luciferase activity (29).

Ethical considerations

All animal experiments were approved by the local authority Animal Care and Use Committee and local ethics committee of the Radboud university medical center (RU-DEC 2011-101/2012-247) and were performed by personnel certified by the Dutch Ministry of WVC.

Statistical analysis

Data were statistically evaluated using the Mann-Whitney U test or student t-test using Graph Pad Prism 5 (GraphPad Software). Differences were called significant with P-value < 0.05 (*), < 0.01 (**) or <0.0005(***).

Results

ASC treatment has no effect on development of joint destruction in DMM experimental OA with low synovial activation

In a previous study we found that a single injection of ASCs into a mouse knee joint of CIOA inhibited development of cartilage destruction, chondrogenesis in ligaments and osteophyte formation (23). CIOA is characterized by thickening

and activation of the synovial layer containing activated macrophages. To investigate further whether synovial macrophage activation may drive the anti-inflammatory activity of ASCs, ASCs were tested in the DMM model, in which synovial macrophage activation is scant (11). A single injection of 20.000 ASCs was given into the right knee joint either at day 7 or day 14 after induction of DMM. In contrast to CIOA, no effect of ASC treatment was found in DMM on cartilage destruction (Figure 1A), osteophyte formation (Figure 1B) or chondrogenesis in ligaments (Figure 1C). These results suggest that the healing capacity of ASC is related to synovitis.

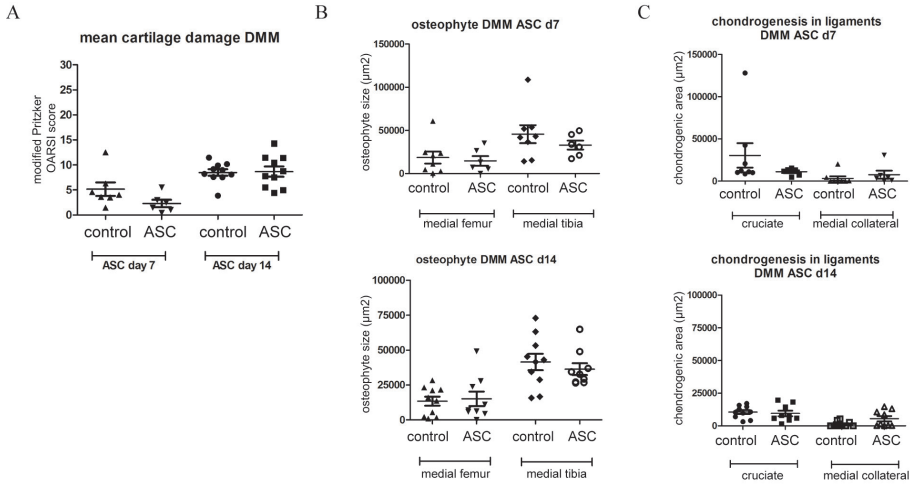

Figure 1: ASC treatment has no effect on development of joint destruction in a DMM model in which synovial activation is scant.
DMM OA was induced by transection of the medial anterior meniscotibial ligament and 20.000 ASCs were injected intra-articularly at day 7 or 14. Mice were sacrificed at day 56 and whole knee joints were prepared for histology. **A**. No difference was found on mean cartilage damage as assessed by the modified Pritzker OARSI score. **B**. Injection of ASCs on neither day 7 or 14 changed osteophyte size as measured at the margins of the medial femur and tibia by image analysis software. **C**. Similarly, chondrogenic area in cruciate and medial collateral ligaments was not changed by ASC injection at day 7 or 14 of DMM, measured by redness in Safranin-O stained sections. N=8 mice/group in experiment with ASC injection at day 7, n=10 at day 14.

Intra-articularly applied ASCs rapidly suppress the activation status of the synovial lining layer during CIOA

To investigate the effect of the intra-articularly injected ASCs on the inflammatory status of the synovium, we injected 20.000 ASCs in the knee joint at day 7 after induction of CIOA and isolated synovium 6 hours, 2, 14 and 42 days thereafter. Various macrophage derived cytokines (IL-1β, IL-6, TNFα, IL-10, KC, S100A8,

S100A9) were measured using RT-qPCR. Pro-inflammatory mediators S100A8, S100A9, IL-1β, IL-6 and KC were highest 6 hours after control injection and declined later on, which is in line with earlier studies showing highest synovial activation in early stages of CIOA (11). Interestingly, ASCs significantly downregulated S100A8, S100A9 (Figure 2A), IL-1β and KC mRNA (Figure 2B) already 6 hours after ASC injection. Although mRNA levels were still lower at day 14, this did not reach significance. IL-6 mRNA levels were significantly reduced at day 14, and lower at day 2, although not significantly (Figure 2B).

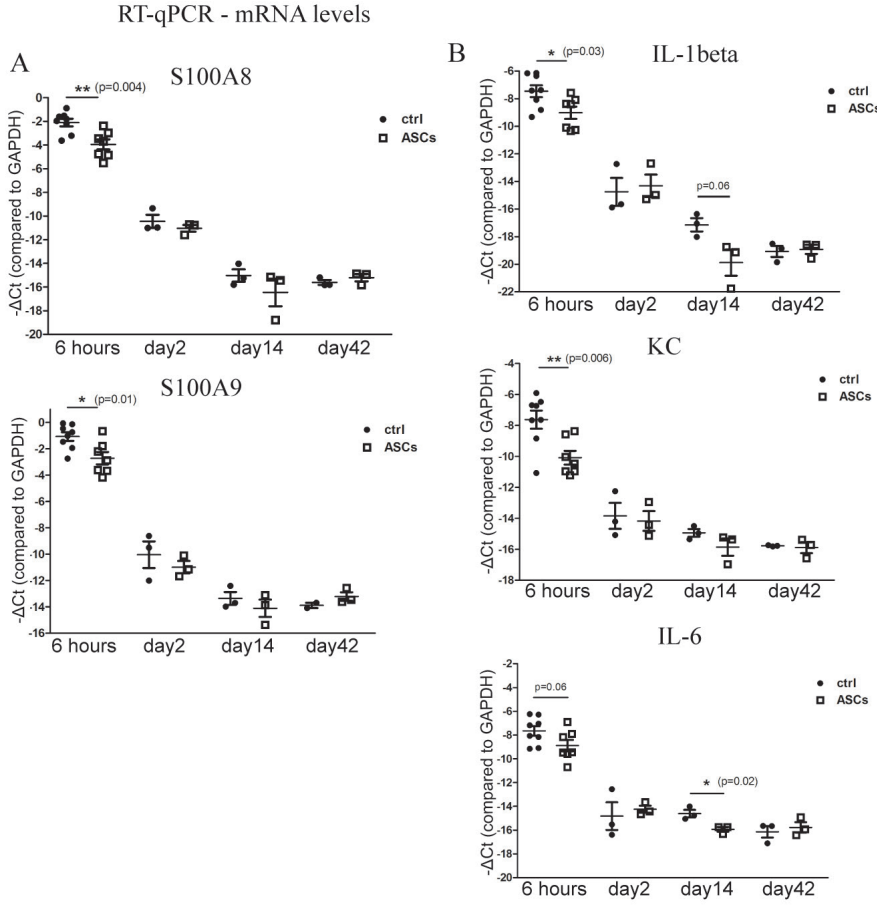

Figure 2: Intra-articularly applied ASCs rapidly suppress synovial mRNA-expression of pro-inflammatory mediators during collagenase-induced OA
ASCs were injected at day 7 of CIOA, and 6 hours, 2, 14 and 42 days thereafter synovial RNA was collected. mRNA levels of alarmins S100A8, A9 (**A**) and pro-inflammatory cytokines/ chemokines IL-1β, IL-6 and KC (**B**) were measured on RT-qPCR and were significantly lowered after 6 hours (except for IL-6), however not at later timepoints. N=6-8 mice/ group (2 synovia pooled at timepoints 2,14,42 days). *=P<0.05, **=P<0.01 as measured by student t-test.

In addition, protein levels of various cytokines (IL-1β, IL-6, IL-10, KC, IFNγ) and S100A8/A9 were measured in synovial washouts using Luminex or ELISA (for S100A8/A9). Low levels of IL-1β (Figure 3B) and very high levels of S100A8/A9 (Figure 3A) were detected 6 hours and 2 days after ASC or control injection. IL-1β protein levels were found to be lower at day 2, 14 and 42 after ASC treatment (44, 45 and 34% lower, respectively) (Figure 3B). Moreover, S100A8/A9 was significantly downregulated on protein level 6 and 48 hours after ASC injection (48 and 58% lower, respectively) (Figure 3A). No effect of ASC treatment was found on protein levels of IL-6 and KC (Figure 3B). These results indicate that the suppressive effect by ASCs is rapidly initiated in the synovium. Apart from lowering catabolic cytokines, ASC treatment may also affect anabolic growth factors like TGFβ and BMP-2, crucial during osteophytosis (30). However, no effect of ASCs was found on mRNA levels of TGFβ and BMP-2 (data not shown). Measuring TGFβ activity in synovial washouts using a CAGA-luc assay, no differences were found at 6 hours, 2, 14 or 42 days after ASC and control treatment (Figure 3C).

Interaction between cytokine stimulated ASCs and macrophages

In various studies it has been shown that pro-inflammatory cytokines, and in particular IFNγ, directly stimulate the anti-inflammatory capacity of ASCs (31-33). First, we confirmed that IFNγ could directly induce anti-inflammatory mediators arginase, IL-1RA and iNOS in ASCs on mRNA level (Figure 4A).IL-1β also induced higher levels of arginase and iNOS mRNA in ASCs, whereas S100A8 did not affect any anti-inflammatory genes in ASCs (Figure 4A). We next studied the in vitro effect of IL-1β and IFNγ on the cross talk between ASCs and activated macrophages. To do this, murine bone marrow derived macrophages (BMM) were activated with LPS and consequently stimulated with supernatant of IL-1β- or IFNγ-stimulated ASCs. The supernatant of IFNγ-stimulated, but not IL-1β-stimulated ASCs strongly increased IDO mRNA expression in BMM (25-fold increase compared to unstimulated ASCs or IFNγ alone), while also upregulating iNOS (83- or 6-fold increase compared to unstimulated ASCs or IFNγ alone). Supernatant of unstimulated ASCs already upregulated arginase mRNA levels in BMM (8-fold increase). No effect was found on IL-1RA, IL-10 and TGFβ mRNA levels (Figure 4B).

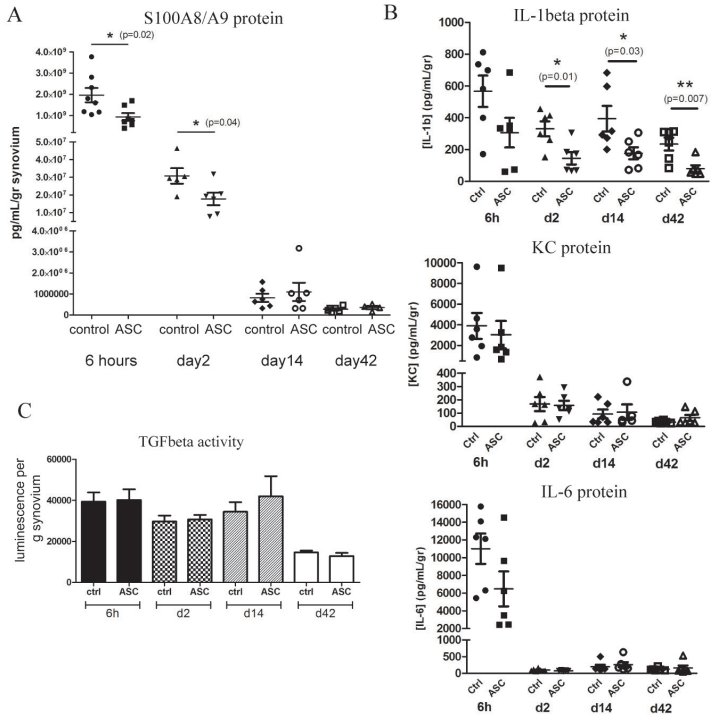

Figure 3: Intra-articular ASC-injection reduces synovial protein levels of pro-inflammatory S100A8/A9 and IL-1β during collagenase-induced OA
ASCs (20.000) were injected at day 7 of CIOA, and 6 hours, 2, 14 and 42 days thereafter synovial washouts were collected. Protein levels of S100A8/A9 were measured with a specific ELISA (**A**) and IL-1β, IL-6 and KC proteins levels were measured on Luminex (**B**). S100A8/A9 levels were significantly lowered 6 hours and 2 days after ASC treatment, IL-1β at day 2, 14 and 42. **C**. TGFβ-activity, measured by CAGA-luc in synovial washouts, was not changed after ASC treatment. N=6 mice/group *=P<0.05, **=P<0.01 as measured by student t-test.

The efficacy of local ASC treatment in the early phase of OA is related to synovial thickness and is reflected by S100A8/A9 levels in the serum

To investigate whether the efficacy of ASC treatment is related to synovial thickness, we compared CIOA with high and somewhat lower synovial inflammation by injecting 1U collagenase either once or twice. The synovial thickening of CIOA on day 42 with one time injection was 65% lower compared to two times injection (2.48 vs 1.5). However, this is still higher than observed in the DMM model (synovial activation score at endpoint, day 56: 0.7, data not shown). In the experiment with high synovial activation, day 7 ASC treatment

significantly suppressed synovial thickening (32% lower). In addition, osteophyte size was also greatly reduced in the ASC treated group (72% lower) (Figure 5A). In contrast, in the second experiment, no suppressive effect of ASC treatment on synovial thickening, nor on osteophyte size (Figure 5B) was found. These data suggest that the inflammatory status of the synovium drives the anti-inflammatory capacity of the ASC.

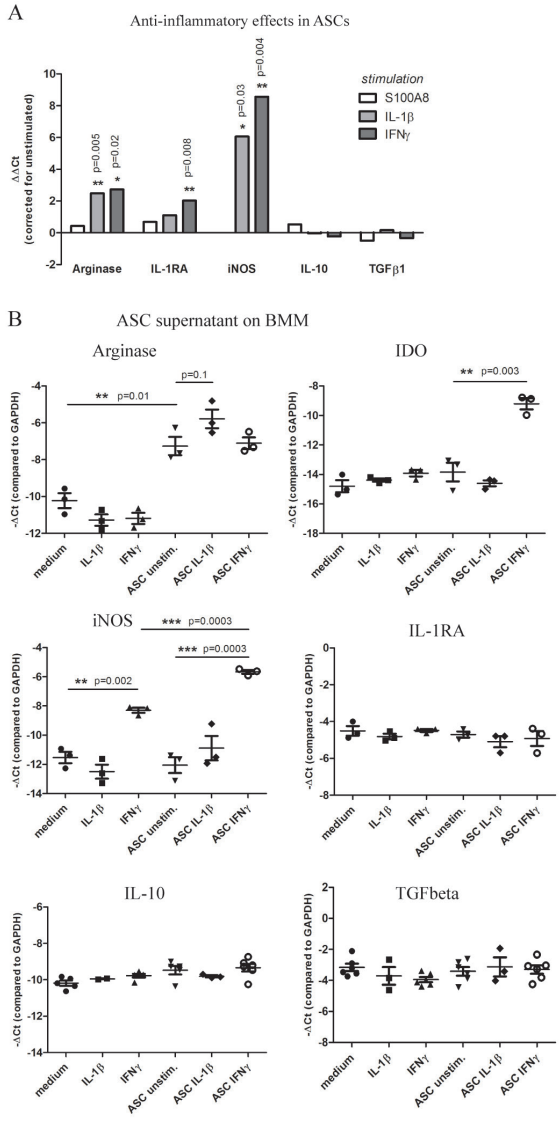

Figure 4: Cytokine stimulated ASCs induce anti-inflammatory effects in activated macrophages
ASCs were stimulated with pro-inflammatory cytokines IFNγ, IL-1β and S100A8 and the supernatants were used to stimulate activated murine bone-marrow derived macrophages (BMM). **A**. Direct effects of IFNγ, IL-1β and S100A8 on the mRNA expression of anti-inflammatory mediators by ASCs, showing upregulation of arginase, IDO, IL-1RA and iNOS. **B**. Supernatants of unstimulated ASCs already upregulated arginase mRNA in BMM, while supernatant of IFNγ-stimulated ASCs upregulated IDO and iNOS mRNA. IL-1RA, IL-10 and TGFβ mRNA levels in BMM were not affected. Values in **A** are represented as ΔΔCt, corrected for GAPDH and unstimulated. Values in **B** are represented as −ΔCt, corrected for GAPDH. Results are representative of 2 independent experiments. * = P < 0.05, ** = P < 0.01, ***=P<0.005 as measured by student t-test.

During synovitis release of high amounts of pro-inflammatory and catabolic S100A8/A9 may leak from the joint into the bloodstream (11). Serum was isolated at 14 and 42 days after ASC-treatment in the two CIOA experiments differing in synovial activation. In the experiment with high synovial activation, serum levels of S100A8/A9 levels were very high at day 14 (497 ng/ml, 371% of non-arthritic C57Bl6 mice, "naïve") and at day 42 still clearly above naïve (282 vs 134 ng/ml) (Figure 6A). In the experiment showing less synovial thickening, serum levels of S100A8/A9 levels were much lower at day 14 (272 versus 497 ng/ml) and returned to control levels at day 42 (Figure 6B). Interestingly, in the first experiment, local application of ASC at day 7 after OA induction dramatically inhibited S100A8/A9 serum levels (80% at day 14 and 44% at day 42 after treatment) (Figure 6A). In contrast, in the experiment with low synovial activation, S100A8/A9 levels were not significantly changed by ASC treatment at day 14 whereas at day 42 a 58% decrease was observed (Figure 6B).

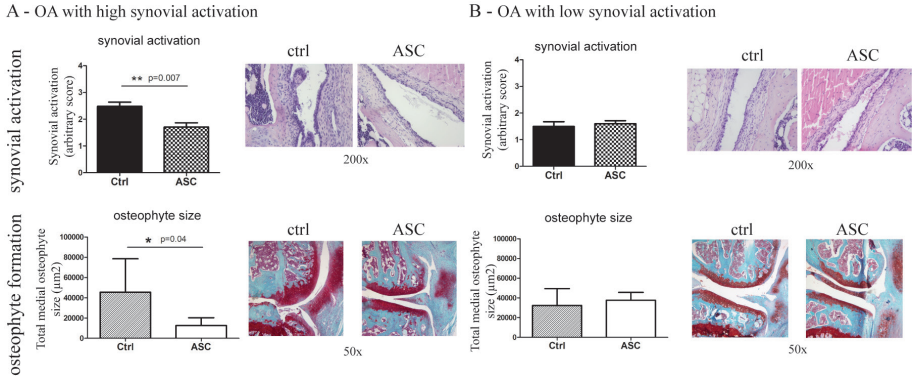

Figure 5 : The efficacy of local ASC treatment in the early phase of collagenase-induced OA depends on synovial activation
Two collagenase-induced OA experiments differing in synovial thickening/activation (1.7 fold difference) at day 42 were compared on the effect of intra-articular ASC treatment at day 7. Local ASC treatment only reduces synovial activation and osteophyte formation at the medial femur in OA with high synovial activation (**A**), compared to OA with low synovial activation (**B**). Right panels show representative histological pictures of hematoxylin-eosin stained knee sections (synovial activation, top panels) or Safranin-O stained knee sections on the medial side (osteophyte formation, bottom panels). Magnification, 50x or 200x, is indicated. *=P-value <0.05, **=P-value<0.01. Significance of osteophyte size was calculated using student t-test, synovial activation with Mann Whitney U. n=6 mice/group

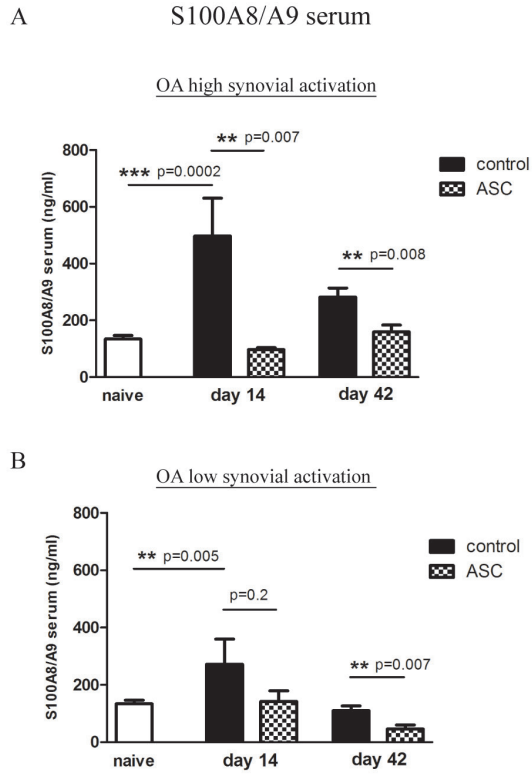

Figure 6: Efficacy of ASC treatment can be measured by serum levels of S100A8/A9
Systemic S100A8/A9 levels in collagenase-induced OA differing in synovial activation was compared and the effect of ASC treatment at day 7 examined. **A**. Serum S100A8/A9 levels were very high in OA with higher synovial activation (807 ng/ml at day 14, compared to 134 ng/ml in non-arthritic mice "naïve") and were significantly lowered at day 14 and 42 when ASCs were given intra-articularly at day 7. **B**. In contrast, S100A8/A9 serum levels were much lower in the experiment with low synovial activation (272 vs 807 ng/ml at day 14). Treatment with ASCs did not significantly decrease S100A8/A9 levels at day 14, only at day 42. S100A8/A9 was measured using ELISA. n=8 mice/group, except naive (n=32) *=P<0.05, **=P<0.01 as measured by student t-test

Discussion

In the present study we find that synovitis is essential in mediating the immunosuppressive effects of ASCs in experimental OA. S100A8/A9, as marker of this synovitis, could be used as predictor for successful ASC-treatment. Furthermore, locally administered ASCs rapidly suppress the activation status of the synovial lining layer possibly via a suppressive effect on macrophages.

Thickening of the synovial lining layer is a characteristic feature of CIOA, paralleling large subgroups of OA patients that show synovitis (1). The synovial layer comprises mainly activated macrophages and selective elimination of these macrophages prior to induction of CIOA strongly inhibited synovial thickening, chondrogenesis/osteophyte formation and cartilage destruction (8). However,

elimination of lining macrophages in DMM, in which synovitis is scant, has no effect on OA pathology. In the present study we find that when ASCs were injected into a DMM OA joint, no effect was found on development of joint destruction. Furthermore, ASC treatment is most efficient when given in a CIOA with high, not low synovitis. These data further support the importance of the activated synovial lining and macrophages in mediating the anti-inflammatory ASC effect.

ASCs injected during the first phase of CIOA encounter high levels of pro-inflammatory cytokines released by activated synovial lining macrophages. During early phase CIOA, levels of IL-1β and S100A8/A9 are strongly upregulated in the synovium (11). In the present study we find that injection of ASCs into day 7 CIOA joints caused a rapid downregulation of both IL-1β and S100A8/A9 levels and this effect was prolongated up to day 42 after treatment (for IL-1β). Also, five days after injection, no ASCs could be detected anymore within the synovium (data not shown). Apparently, a short pulse is sufficient for giving a prolonged ameliorating effect. An explanation may be that the ASC imprint on synovial macrophages may result in transformation into suppressive macrophages producing lower amounts of IL-1β or S100A8/A9 cytokines.

Although we show clear anti-inflammatory effects of ASCs in CIOA, we cannot rule out that cell-types other than MSCs could also induce immunomodulatory capacities. Indeed, we have shown earlier that skin fibroblasts exert anti-inflammatory effects in collagen-induced arthritis (34). However, we believe that the use of ASCs is superior to other cell-types. First, they have the ability to differentiate into cell-types that can contribute to joint repair, such as chondrocytes and osteoblasts (15). Furthermore, ASCs have an advantage of use in osteoarthritis compared to fibroblasts, because the anti-inflammatory effect of the latter is largely attributed to inhibition of T-cell proliferation and activation with T-cells playing no role in OA pathogenesis. Finally, MSCs, to which ASCs belong, are known for their low immunogenicity and can be easily used in therapeutic approaches, without the need of autologous application (35).

It is becoming increasingly accepted that MSCs secrete many factors including growth factors, cytokines, chemokines, metabolites and bioactive lipids which orchestrate multiple interactions with the surrounding microenvironment (36). Pro-inflammatory cytokines, like IFNγ, IL-6, TNF-α and DAMPs, present during the first phase of CIOA, are capable of inducing an immunosuppressive

phenotype in ASCs/MSCs (17, 19, 20, 32). In reaction to these stimuli, ASCs release factors like IDO, COX-2 and PGE2 which are able to suppress activated macrophages, although subtle species differences exist between mouse and man (17, 37). In line with that we show that ASCs are able to induce expression of anti-inflammatory mediators arginase and iNOS after IL-1β or IFNγ stimulation and upregulate IL-1RA after IFNγ stimulation. Moreover, several in vitro and in vivo studies suggest that MSCs can decrease inflammation by changing the macrophage phenotype from M1 (classically activated) to M2 (alternatively activated) (38-40). Previously, we already found that ASCs, when injected into the knee joint, interact with synovial lining macrophages (23). We now find that supernatant of IFNγ stimulated ASC strongly upregulated arginase, IDO and iNOS in activated macrophages which is characteristic for transformation into a M2 signature.We speculate that both IL-1β and IFNγ could stimulate ASCs in vivo during (experimental) OA, thereby activating their anti-inflammatory phenotype, possibly via macrophages. IL-1β is frequently studied and detected in OA synovium (41, 42). Although data on IFNγ in OA is more scarce, it has been shown to be present in OA synovium by immunohistochemistry (41, 43) and IFNγ positive CD4 T-cells were found in OA synovium (44, 45). Finally, IFNγ (and to a lesser extent IL-1β) could also be used as tool to activate the ASCs ex vivo to generate a larger anti-inflammatory potential.

Chondrogenesis within collateral/cruciate ligaments hampers a good functioning of the joint and may further enhance cartilage destruction. In previous studies performed in CIOA, we showed that activated synovial macrophages mediate chondrogenesis/osteophyte formation (9, 46). Macrophage derived factors involved in new cartilage formation include growth factors like TGFβ and BMP-2 (9, 47). Production of these growth factors within the synovium was strongly diminished after macrophages were selectively depleted from the lining layer (46). However, ASC treatment did not suppress mRNA levels of TGFβ and BMPs or active TGFβ within the synovium, suggesting that ASCs mostly act via inhibition of pro-inflammatory cytokines (IL-1β) or alarmins S100A8/A9, rather than via growth factors.

Comparing efficacy of ASC treatment in two CIOA experiments differing in synovial activation we found that ASC treatment was anti-inflammatory particularly when injected in joints with high but not with low synovitis, indicating that a certain threshold of pro-inflammatory factors is needed for induction of the anti-

inflammatory effect of ASCs. In line with this is that ASC treatment when given at later phases (day 14 or day 21) after CIOA is also not effective (23) and that treatment of DMM with ASCs is also ineffective (Figure 1). High synovial activation in CIOA joints was reflected by high S100A8/A9 levels in the serum (Figure 6). S100A8/A9 produced in the synovium could leak out of the joint cavity thereby reflecting inflammatory status in the serum (48). S100A8/A9 serum levels may thus be used firstly as a marker for determining synovitis which is a licensing signal for using local ASC treatment. Secondly, local stem cell treatment of CIOA joints lowered levels of S100A8/A9 levels within the serum and may therefore also be used as a marker for measuring the efficacy of ASC treatment.

In the present paper we show that intra-articular deposition of ASCs inhibit thickening/activation of the synovial lining layer and protect joint destruction in experimental OA with high synovial activation. ASCs had an immunomodulatory effect on the synovium by suppressing catabolic factors like IL-1β and S100A8/A9 produced by synovial macrophages. In vitro, ASCs upregulated anti-inflammtory factors in activated macrophages. Finally, S100A8/A9 could be used both as marker to determine feasibility of ASC-treatment in experimental OA as well as read-out to determine its efficacy.

ASCs may be a potent and safe tool to combat joint destruction and inflammation in OA patients with high synovial activation.

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References

1. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nature reviews Rheumatology. 2011;7(1):33-42.

2. Wang Q, Rozelle AL, Lepus CM, Scanzello CR, Song JJ, Larsen DM, et al. Identification of a central role for complement in osteoarthritis. Nature medicine. 2011;17(12):1674-9.

3. Haywood L, McWilliams DF, Pearson CI, Gill SE, Ganesan A, Wilson D, et al. Inflammation and angiogenesis in osteoarthritis. Arthritis and rheumatism. 2003;48(8):2173-7.

4. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis -- results of a 1 year longitudinal arthroscopic study in 422 patients. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2005;13(5):361-7.

5. Iagnocco A, Coari G. Usefulness of high resolution US in the evaluation of effusion in osteoarthritic first carpometacarpal joint. Scandinavian journal of rheumatology. 2000;29(3):170-3.

6. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Annals of the rheumatic diseases. 2005;64(9):1263-7.

7. Bondeson J, Blom AB, Wainwright S, Hughes C, Caterson B, van den Berg WB. The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis. Arthritis and rheumatism. 2010;62(3):647-57.

8. Blom AB, van Lent PL, Libregts S, Holthuysen AE, van der Kraan PM, van Rooijen N, et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. Arthritis and rheumatism. 2007;56(1):147-57.

9. van Lent PL, Blom AB, van der Kraan P, Holthuysen AE, Vitters E, van Rooijen N, et al. Crucial role of synovial lining macrophages in the promotion of transforming growth factor beta-mediated osteophyte formation. Arthritis and rheumatism. 2004;50(1):103-11.

10. Chan JK, Roth J, Oppenheim JJ, Tracey KJ, Vogl T, Feldmann M, et al. Alarmins: awaiting a clinical response. The Journal of clinical investigation. 2012;122(8):2711-9.

11. van Lent PL, Blom AB, Schelbergen RF, Sloetjes A, Lafeber FP, Lems WF, et al. Active involvement of alarmins S100A8 and S100A9 in the regulation of synovial activation and joint destruction during mouse and human osteoarthritis. Arthritis and rheumatism. 2012;64(5):1466-76.

12. Schelbergen RF, Blom AB, van den Bosch MH, Sloetjes A, Abdollahi-Roodsaz S, Schreurs BW, et al. Alarmins S100A8 and S100A9 elicit a catabolic effect in human osteoarthritic chondrocytes that is dependent on Toll-like receptor 4. Arthritis and rheumatism. 2012;64(5):1477-87.

13. Maumus M, Guerit D, Toupet K, Jorgensen C, Noel D. Mesenchymal stem cell-based therapies in regenerative medicine: applications in rheumatology. Stem cell research & therapy. 2011;2(2):14.

14. Coleman CM, Curtin C, Barry FP, O'Flatharta C, Murphy JM. Mesenchymal stem cells and osteoarthritis: remedy or accomplice? Human gene therapy. 2010;21(10):1239-50.

15. O'Sullivan J, D'Arcy S, Barry FP, Murphy JM, Coleman CM. Mesenchymal chondroprogenitor cell origin and therapeutic potential. Stem cell research & therapy. 2011;2(1):8.

16. Pittenger M. Sleuthing the source of regeneration by MSCs. Cell stem cell. 2009;5(1):8-10.

17. Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nature medicine. 2009;15(1):42-9.

18. Maggini J, Mirkin G, Bognanni I, Holmberg J, Piazzon IM, Nepomnaschy I, et al. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. PloS one. 2010;5(2):e9252.

19. Bouffi C, Bony C, Courties G, Jorgensen C, Noel D. IL-6-dependent PGE2 secretion by mesenchymal stem cells inhibits local inflammation in experimental arthritis. PloS one. 2010;5(12):e14247.

20. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. European journal of immunology. 2008;38(6):1745-55.

21. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, et al. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. British journal of haematology. 2005;129(1):118-29.

22. De Toni F, Poglio S, Youcef AB, Cousin B, Pflumio F, Bourin P, et al. Human adipose-derived stromal cells efficiently support hematopoiesis in vitro and in vivo: a key step for therapeutic studies. Stem cells and development. 2011;20(12):2127-38.

23. ter Huurne M, Schelbergen R, Blattes R, Blom A, de Munter W, Grevers LC, et al. Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. Arthritis and rheumatism. 2012;64(11):3604-13.

24. van der Kraan PM, Vitters EL, van Beuningen HM, van de Putte LB, van den Berg WB. Degenerative knee joint lesions in mice after a single intra-articular collagenase injection. A new model of osteoarthritis. Journal of experimental pathology. 1990;71(1):19-31.

25. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2007;15(9):1061-9.

26. Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, et al. Osteoarthritis cartilage histopathology: grading and staging. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2006;14(1):13-29.

27. van der Kraan PM, Vitters EL, van de Putte LB, van den Berg WB. Development of osteoarthritic lesions in mice by "metabolic" and "mechanical" alterations in the knee joints. The American journal of pathology. 1989;135(6):1001-14.

28. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nature medicine. 2007;13(9):1042-9.

29. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. The EMBO journal. 1998;17(11):3091-100.

30. Blaney Davidson EN, Vitters EL, van Beuningen HM, van de Loo FA, van den Berg WB, van der Kraan PM. Resemblance of osteophytes in experimental osteoarthritis to transforming growth factor beta-induced osteophytes: limited role of bone morphogenetic protein in early osteoarthritic osteophyte formation. Arthritis and rheumatism. 2007;56(12):4065-73.

31. van Lent PL, van den Berg WB. Mesenchymal stem cell therapy in osteoarthritis: advanced tissue repair or intervention with smouldering synovial activation? Arthritis research & therapy. 2013;15(2):112.

32. Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Pescatori M, Stubbs AP, et al. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clinical and experimental immunology. 2010;162(3):474-86.

33. Leto Barone AA, Khalifian S, Lee WP, Brandacher G. Immunomodulatory effects of

adipose-derived stem cells: fact or fiction? BioMed research international. 2013;2013:383685.

34. Bouffi C, Bony C, Jorgensen C, Noel D. Skin fibroblasts are potent suppressors of inflammation in experimental arthritis. Annals of the rheumatic diseases. 2011;70(9):1671-6.

35. Ghannam S, Bouffi C, Djouad F, Jorgensen C, Noel D. Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications. Stem cell research & therapy. 2010;1(1):2.

36. Saether EE, Chamberlain CS, Leiferman EM, Kondratko-Mittnacht JR, Li WJ, Brickson SL, et al. Enhanced Medial Collateral Ligament Healing Using Mesenchymal Stem Cells: Dosage Effects on Cellular Response and Cytokine Profile. Stem cell reviews. 2013.

37. Mitsui H, Aoyama T, Furu M, Ito K, Jin Y, Maruyama T, et al. Prostaglandin E2 receptor type 2-selective agonist prevents the degeneration of articular cartilage in rabbit knees with traumatic instability. Arthritis research & therapy. 2011;13(5):R146.

38. Cho DI, Kim MR, Jeong HY, Jeong HC, Jeong MH, Yoon SH, et al. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. Experimental & molecular medicine. 2014;46:e70.

39. Nakajima H, Uchida K, Guerrero AR, Watanabe S, Sugita D, Takeura N, et al. Transplantation of mesenchymal stem cells promotes an alternative pathway of macrophage activation and functional recovery after spinal cord injury. Journal of neurotrauma. 2012;29(8):1614-25.

40. Dayan V, Yannarelli G, Billia F, Filomeno P, Wang XH, Davies JE, et al. Mesenchymal stromal cells mediate a switch to alternatively activated monocytes/macrophages after acute myocardial infarction. Basic research in cardiology. 2011;106(6):1299-310.

41. de Lange-Brokaar BJ, Ioan-Facsinay A, van Osch GJ, Zuurmond AM, Schoones J, Toes RE, et al. Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2012;20(12):1484-99.

42. Scanzello CR, Umoh E, Pessler F, Diaz-Torne C, Miles T, Dicarlo E, et al. Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2009;17(8):1040-8.

43. Ishii H, Tanaka H, Katoh K, Nakamura H, Nagashima M, Yoshino S. Characterization of infiltrating T cells and Th1/Th2-type cytokines in the synovium of patients with osteoarthritis. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2002;10(4):277-81.

44. Yamada H, Nakashima Y, Okazaki K, Mawatari T, Fukushi J, Oyamada A, et al. Preferential accumulation of activated Th1 cells not only in rheumatoid arthritis but also in osteoarthritis joints. The Journal of rheumatology. 2011;38(8):1569-75.

45. Yudoh K, Matsuno H, Nakazawa F, Yonezawa T, Kimura T. Reduced expression of the regulatory CD4+ T cell subset is related to Th1/Th2 balance and disease severity in rheumatoid arthritis. Arthritis and rheumatism. 2000;43(3):617-27.

46. Blom AB, van Lent PL, Holthuysen AE, van der Kraan PM, Roth J, van Rooijen N, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2004;12(8):627-35.

47. Scharstuhl A, Vitters EL, van der Kraan PM, van den Berg WB. Reduction of osteophyte formation and synovial thickening by adenoviral overexpression of transforming growth factor beta/bone morphogenetic protein inhibitors during experimental osteoarthritis. Arthritis and rheumatism. 2003;48(12):3442-51.

48. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. Journal of leukocyte biology. 2009;86(3):557-66.

Chapter 9

Summary and final considerations

Chapter 9: Summary and final considerations

The aims of this thesis were to broaden our knowledge on the role of synovial activation and the resulting DAMPs or alarmins S100A8 and S100A9 in OA and to assess efficacy of dampening synovial activation in OA by either inhibiting S100A8/A9 or making use of anti-inflammatory ASCs.

S100A8/A9 plays pivotal role in pathology of experimental OA with synovial activation

Since very little is known regarding the involvement of alarmins S100A8 and S100A9 in osteoarthritis, we started by studying the expression of S100A8 and S100A9 during experimental OA (Chapter 2). To do this, we made use of two murine OA models: CIOA is induced by intra-articular injection of collagenase and synovial thickening is a common feature of this model (1). In contrast, synovial activation is nearly absent in the DMM model, that is induced by transection of the medial anterior meniscotibial ligament (2). We found that S100A8 and S100A9 were greatly induced during CIOA up to day 21, while other pro-inflammatory mediators like IL-1β and TNFα already waned after day 7. In the DMM model, S100A8 and S100A9 were expressed at much lower levels compared to CIOA and only elevated at day 7. When we induced CIOA in the S100A9 -/- mouse, synovial thickening and cartilage destruction were significantly reduced at the end point compared to CIOA in wild-types. In sharp contrast, synovitis, already low in DMM, was not further reduced in S100A9 -/- mice and cartilage destruction was not different between S100A9 -/- and wild-type mice.

Next to cartilage damage, osteophyte formation is an important pathological feature of OA. Osteophytes are fibro-cartilage capped bony outgrowths that can limit joint movement and cause pain. In Chapter 3 we investigated the role of DAMPs S100A8 and S100A9 in osteophyte formation during OA. We assessed osteophyte development in S100A9 -/- mice after induction of CIOA and DMM OA. At the endpoint of DMM, osteophyte size was not different between knockout and wild-type animals. In contrast, osteophyte size was dramatically reduced in

S100A9 -/- mice at the endpoint and halfway during CIOA at multiple locations in the joint. These results, combined with the observed reduction in cartilage damage in Chapter 2, emphasize the importance of synovial activation and the resulting S100A8/A9 production in the development of pathology in OA.

High S100A8/A9 levels in early symptomatic human OA predict progression of disease

Next, we zoomed in on the relationship between S100A8/A9 and human OA in Chapter 2 and 3. To do this, we used the CHECK cohort of symptomatic early OA patients. Synovial mRNA of S100A8 and S100A9 was increased in both early symptomatic and end-stage OA compared to healthy synovium as were protein levels which was confirmed by immunohistochemistry. This again suggests prolonged expression and release of DAMPs S100A8/A9 during OA. Then we measured systemic S100A8/A9 in the blood of CHECK participants at baseline with ELISA and measured joint destruction (according to Kellgren/Lawrence scale) after 2 years (Chapter 2) and osteophyte size after 2 and 5 years (Chapter 3). Participants that had no joint damage at baseline, but developed joint damage after 2 years (Kellgren/Lawrence score ≥1) had significantly higher S100A8/A9 levels at baseline. In line with this, participants that progressed in osteophyte size after 2 or 5 years, also had significantly increased S100A8/A9 levels at entry in the CHECK. Interestingly, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) or Cartilage Oligomeric Matrix Protein (COMP) could not predict osteophyte progression. Together, these results strongly recommend S100A8/A9 as early biomarker for the development of OA. Patient populations used in this study were still fairly small though (n=177 and 56 for joint damage and osteophyte size, respectively) and validation in a larger cohort is necessary. Possibly, the predictive value of S100A8/A9 for OA pathology will be strongest in patients with concurring synovial inflammation as this largely drives S100A8/A9 production and S100A8/A9 is already being used as biomarker in multiple inflammatory diseases (3).

S100A8/A9 exerts pro-inflammatory and catabolic effects on chondrocytes and M1- and M2-macrophages

Endogenous DAMPs, like S100A8 and S100A9, have different functions in- and outside of the cell. Inside they often have a homeostatic role, while secreted they serve as danger signals, hence the term alarmins. In Chapter 4, we stimulated chondrocytes from end-stage OA patients with S100A8 and S100A9 and found that pro-inflammatory (cytokines) and catabolic (MMPs) factors were significantly upregulated on mRNA expression and protein release. Moreover, expression of anabolic genes aggrecan and collagen type II were significantly down-regulated, in combination leading to a shift towards a more catabolic chondrocyte. Interestingly, we showed that this catabolic skewing is specific for osteoarthritic chondrocytes, not normal chondrocytes and appeared to be TLR4 dependent, not RAGE or n-glycans. This is in line with studies showing TLR4 as receptor for S100A8/A9 in monocytes/macrophages and osteoclasts (4, 5).

In Chapter 5, we shifted our attention to the effects of S100A8 and S100A9 on the OA synovium and cells residing within this tissue. The synovial intima layer contains both macrophages and fibroblast-like cells. Fahy et al. recently showed that OA synovium contains both pro-inflammatory M1- and anti-inflammatory M2-macrophages and we could confirm this in our OA synovial explants (6). First, we proved that S100A9 could significantly upregulate pro-inflammatory cytokines (MMPs in lesser extent) in total OA synovial explants. Then we focused on specific effects on the different cell-types of the synovium. We could not find effects of S100A9 on synovial fibroblasts. However, S100A9 had clear pro-inflammatory effects on both M1- and M2-macrophages, with M2-macrophages being slightly less responsive. These results suggest that S100A8/A9, abundantly present during synovitis, increases pro-inflammatory and catabolic mediators through both M1- and M2-macrophages.

Blocking S100A8/A9 with paquinimod alleviates experimental OA pathology

Although OA affects millions of people worldwide and their number is increasing due to the increasing age and obesity of the population, still no disease modifying

OA drugs have been developed yet. In the last chapters we explored the possibility to treat experimental OA by modulating synovitis. In Chapter 6, we used an inhibitor of S100A9, the quinoline derivative paquinimod, that specifically inhibits the binding of S100A9 with its receptors TLR4 and RAGE (7). This compound has already proven to be effective in experimental lupus and encephalomyelitis (8, 9) and we now show disease modifying effects for the first time in experimental OA. Paquinimod administered a few days prior to induction of CIOA significantly reduced synovial thickening, osteophyte size and cartilage damage at the end point. In line with our studies in the S100A9 -/- mice, blocking S100A9 with paquinimod in DMM OA did not have any effects on cartilage damage and only slightly on osteophyte formation. Interestingly, we also show that paquinimod can block the induction of pro-inflammatory and catabolic mediators by S100A9 in ex vivo stimulations with human OA synovium. These findings, combined with the fact that paquinimod has already proven to be safe and tolerable (8), provide us with a feasible option for future treatment of inflammatory type OA.

Adipose-derived stem cells dampen synovial activation and reduce pathology in experimental OA

Synovial inflammation can alternatively be blocked by the use of adipose-derived pluripotent stem cells (ASCs). These cells like their close relatives, bone marrow derived mesenchymal stem cells (BM MSCs), have been shown to have strong anti-inflammatory characteristics. They can produce numerous anti-inflammatory factors and BM MSCs and ASCs have been used in a wide range of inflammatory or auto-immune diseases (10, 11). In Chapter 7, we have successfully used ASCs in an OA setting. ASCs intra-articularly injected 7 days after induction of CIOA significantly reduced synovial thickening, new cartilage and bone formation (osteophytes) in ligaments and cartilage damage at the end point. We could visualize ASCs no longer than 1 day after injection, in close contact with macrophages in the synovial lining layer. As ASC treatment was only successful in the presence of inflammation, this suggested involvement of the synovium in driving the anti-inflammatory mechanism of the ASCs and in Chapter 8, we analyzed changes in expression and production of cytokines in the synovium. We found that ASCs could rapidly (already after 6 hours) downregulate pro-inflammatory cytokines/chemokines and DAMPs S100A8 and S100A9, products

of activated macrophages. Next, we showed that synovial activation is essential for the efficacy of the ASCs, as CIOA with low synovial inflammation and DMM OA could not be treated efficiently with day 7 injection of ASCs. Finally, we measured S100A8/A9 during and after treatment of CIOA with ASCs and showed that the levels of these DAMPs reflect efficient treatment.

Final considerations

By using sensitive magnetic resonance imaging (MRI) and ultra-sound techniques, it has become apparent that inflammation is very common in both knee and hand OA (12, 13). OA therapy is currently focused on treating pain and relieving symptoms, instead of treating the cause of the disease. Non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids are commonly used to dampen inflammation and alleviate pain during OA, but these are broad-spectrum approaches and have lots of side effects. We have shown that paquinimod, a quinoline-3-carboxamide compound (Q-compound), effectively reduces synovial activation, cartilage damage and osteophyte formation in experimental OA. Paquinimod inhibits binding of S100A9 to both its receptors TLR4 and RAGE (7) and may therefore specifically block synovial activation during OA. Moreover, in preclinical studies in systemic lupus erythematosus (SLE), paquinimod has proved safe with little side effects (8). Q-compounds have been studied extensively in the treatment of human auto-immune and inflammatory diseases (14, 15), while paquinimod is currently in clinical development for systemic sclerosis (16). Interestingly, in several animal models of auto-immune / inflammatory diseases Q-compounds have a potent effect on disease pathology, yet do not suppress adaptive immunity (7, 17). In that light Q-compound paquinimod would be suitable for treatment of OA, which has little to no involvement of T- and B-cells, without having severe side effects on the patient's immune system.

A mode of action for paquinimod could be by blocking chemotaxis of myeloid cells. Recently, paquinimod has been shown to inhibit recruitment of inflammatory Ly6C-high monocytes during sterile peritoneal inflammation (18). Possibly this effect could be through blocking of S100A9, as a relation between S100A9 and Ly6C-bearing monocytes has been shown before (19, 20).

Dampening synovial activation locally can be achieved in experimental OA by local injection of ASCs, as we have shown in Chapter 7 and 8. A short pulse of ASCs already seems sufficient to reduce synovial activation and further aggravation of OA development, keeping possible systemic side effects to a minimum. ASCs might achieve these effects by inducing anti-inflammatory mediators like IDO, COX-2 and PGE2 which are able to suppress activated macrophages (21). We and others have shown that these stem cells interact closely with synovial macrophages (Chapter 7, (22)) and several studies suggest that mesenchymal stem cells can skew the macrophage phenotype from pro-inflammatory M1 to alternatively activated M2-macrophages (23, 24).

ASCs or BM MSCs as anti-inflammatory therapy has been used in several experimental inflammatory diseases like arthritis and ischemic heart disease (25, 26) and recently also in an experimental osteoarthritis models in the rabbit (22). Moreover, ASCs could also exert anti-inflammatory effects on human OA chondrocytes and synoviocytes in vitro (27). Together, the anti-inflammatory results so far in experimental and human in vitro models show great promise for the use of ASCs or BM MSCs as therapy in inflammatory OA.

Overall conclusion

In this thesis we have shown that synovial activation and the resulting DAMPs S100A8 and S100A9 are pivotal in the development of OA pathology. Moreover, we show promising experimental data that blocking this synovial activation either directly with ASCs or with paquinimod, blocking S100A8 and S100A9, could be future therapeutic options against inflammatory OA.

References

1. van der Kraan PM, Vitters EL, van Beuningen HM, van de Putte LB, van den Berg WB. Degenerative knee joint lesions in mice after a single intra-articular collagenase injection. A new model of osteoarthritis. J Exp Pathol (Oxford). 1990;71(1):19-31.

2. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. Osteoarthritis Cartilage. 2007;15(9):1061-9.

3. Chan JK, Roth J, Oppenheim JJ, Tracey KJ, Vogl T, Feldmann M, et al. Alarmins: awaiting a clinical response. J Clin Invest. 2012;122(8):2711-9.

4. Grevers LC, de Vries TJ, Vogl T, Abdollahi-Roodsaz S, Sloetjes AW, Leenen PJM, et al. S100A8 enhances osteoclastic bone resorption in vitro through activation of Toll-like receptor 4: implications for bone destruction in murine antigen-induced arthritis. Arthritis Rheum. 2011;63(5):1365-75.

5. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007;13(9):1042-9.

6. Fahy N, de Vries-van Melle ML, Lehmann J, Wei W, Grotenhuis N, Farrell E, et al. Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. Osteoarthritis Cartilage. 2014.

7. Bjork P, Bjork A, Vogl T, Stenstrom M, Liberg D, Olsson A, et al. Identification of human S100A9 as a novel target for treatment of autoimmune disease via binding to quinoline-3-carboxamides. PLoS biology. 2009;7(4):e97.

8. Bengtsson AA, Sturfelt G, Lood C, Ronnblom L, van Vollenhoven RF, Axelsson B, et al. Pharmacokinetics, tolerability, and preliminary efficacy of paquinimod (ABR-215757), a new quinoline-3-carboxamide derivative: studies in lupus-prone mice and a multicenter, randomized, double-blind, placebo-controlled, repeat-dose, dose-ranging study in patients with systemic lupus erythematosus. Arthritis Rheum. 2012;64(5):1579-88.

9. Helmersson S, Sundstedt A, Deronic A, Leanderson T, Ivars F. Amelioration of experimental autoimmune encephalomyelitis by the quinoline-3-carboxamide paquinimod: reduced priming of proinflammatory effector CD4(+) T cells. Am J Pathol. 2013;182(5):1671-80.

10. Maumus M, Guerit D, Toupet K, Jorgensen C, Noel D. Mesenchymal stem cell-based therapies in regenerative medicine: applications in rheumatology. Stem cell research & therapy. 2011;2(2):14.

11. Gonzalez-Rey E, Gonzalez MA, Varela N, O'Valle F, Hernandez-Cortes P, Rico L, et al. Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis. Ann Rheum Dis. 2010;69(1):241-8.

12. Baker K, Grainger A, Niu J, Clancy M, Guermazi A, Crema M, et al. Relation of synovitis to knee pain using contrast-enhanced MRIs. Ann Rheum Dis. 2010;69(10):1779-83.

13. Haugen IK, Boyesen P, Slatkowsky-Christensen B, Sesseng S, van der Heijde D, Kvien TK. Associations between MRI-defined synovitis, bone marrow lesions and structural features and measures of pain and physical function in hand osteoarthritis. Ann Rheum Dis. 2012;71(6):899-904.

14. Kieseier BC. Defining a role for laquinimod in multiple sclerosis. Therapeutic advances in neurological disorders. 2014;7(4):195-205.

15. Kleinau S, Larsson P, Bjork J, Holmdahl R, Klareskog L. Linomide, a new immunomodulatory drug, shows different effects on homologous versus heterologous collagen-

induced arthritis in rats. Clin Exp Immunol. 1989;78(1):138-42.

16. Hesselstrand R, Distler JH, Riemekasten G, Törngren M, Nyhlén HC, Stenström M, et al. An open-label study to evaluate biomarkers and safety in systemic sclerosis (SSc) patients treated with paquinimod (ABR-215757). EULAR 2014. 10.1136/annrheumdis-2014-eular.4207; 2014.

17. Brunmark C, Runstrom A, Ohlsson L, Sparre B, Brodin T, Astrom M, et al. The new orally active immunoregulator laquinimod (ABR-215062) effectively inhibits development and relapses of experimental autoimmune encephalomyelitis. Journal of neuroimmunology. 2002;130(1-2):163-72.

18. Deronic A, Helmersson S, Leanderson T, Ivars F. The quinoline-3-carboxamide paquinimod (ABR-215757) reduces leukocyte recruitment during sterile inflammation: Leukocyte- and context-specific effects. International immunopharmacology. 2014;18(2):290-7.

19. Zhao F, Hoechst B, Duffy A, Gamrekelashvili J, Fioravanti S, Manns MP, et al. S100A9 a new marker for monocytic human myeloid-derived suppressor cells. Immunology. 2012;136(2):176-83.

20. Kallberg E, Vogl T, Liberg D, Olsson A, Bjork P, Wikstrom P, et al. S100A9 interaction with TLR4 promotes tumor growth. PLoS One. 2012;7(3):e34207.

21. Mitsui H, Aoyama T, Furu M, Ito K, Jin Y, Maruyama T, et al. Prostaglandin E2 receptor type 2-selective agonist prevents the degeneration of articular cartilage in rabbit knees with traumatic instability. Arthritis Res Ther. 2011;13(5):R146.

22. Desando G, Cavallo C, Sartoni F, Martini L, Parrilli A, Veronesi F, et al. Intra-articular delivery of adipose derived stromal cells attenuates osteoarthritis progression in an experimental rabbit model. Arthritis Res Ther. 2013;15(1):R22.

23. Cho DI, Kim MR, Jeong HY, Jeong HC, Jeong MH, Yoon SH, et al. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. Experimental & molecular medicine. 2014;46:e70.

24. Dayan V, Yannarelli G, Billia F, Filomeno P, Wang XH, Davies JE, et al. Mesenchymal stromal cells mediate a switch to alternatively activated monocytes/macrophages after acute myocardial infarction. Basic Res Cardiol. 2011;106(6):1299-310.

25. Bouffi C, Bony C, Courties G, Jorgensen C, Noel D. IL-6-dependent PGE2 secretion by mesenchymal stem cells inhibits local inflammation in experimental arthritis. PLoS One. 2010;5(12):e14247.

26. Herrmann JL, Abarbanell AM, Wang Y, Weil BR, Poynter JA, Manukyan MC, et al. Transforming growth factor-alpha enhances stem cell-mediated postischemic myocardial protection. The Annals of thoracic surgery. 2011;92(5):1719-25.

27. Manferdini C, Maumus M, Gabusi E, Piacentini A, Filardo G, Peyrafitte JA, et al. Adipose-derived mesenchymal stem cells exert antiinflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2. Arthritis Rheum. 2013;65(5):1271-81.

Chapter 10

Nederlandse samenvatting
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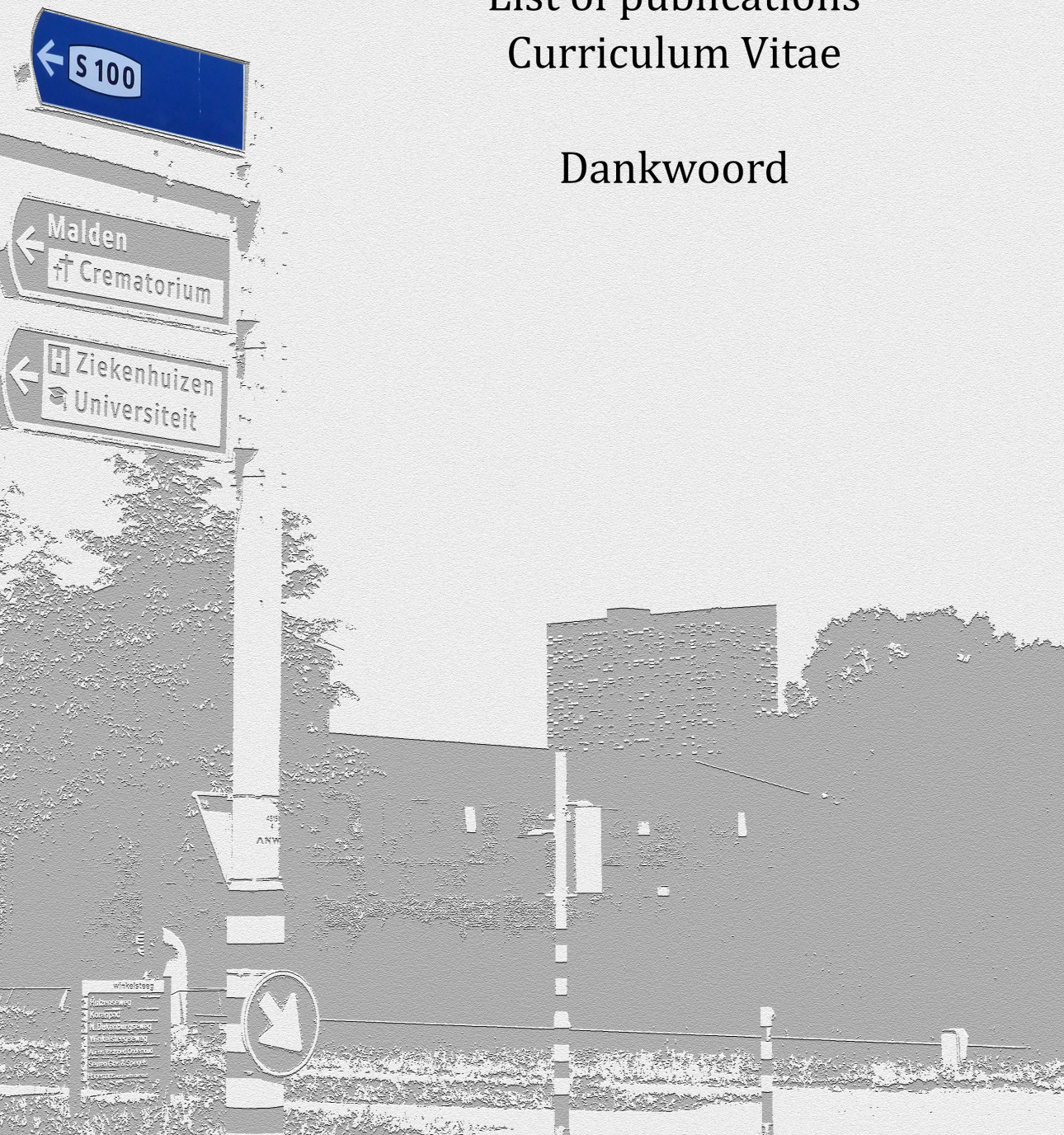
Dankwoord



Chapter 10

Nederlandse samenvatting

Artrose (OA): een groeiend gezondheidsprobleem

Ouderdomsziektes vormen in de huidige Westerse wereld een steeds groter probleem, zowel maatschappelijk als financieel, door de steeds ouder wordende bevolking. Eén van de meest voorkomende ouderdomsziektes is artrose of osteoartritis (afgekort: OA). Meer dan 50% van de bevolking ouder dan 65 heeft een zekere mate van OA die kan worden vastgesteld door een reumatoloog. OA kenmerkt zich door een slijtage van het kraakbeen in gewrichten van met name handen/vingers, ruggenwervel, knie en/of heup. Door het wegvallen van de dempende werking van het kraakbeen ontstaat er direct bot op bot contact wat kan leiden tot gewrichtspijn en verminderde beweeglijkheid van het gewricht. OA kan een zware impact hebben op de kwaliteit van leven, doordat patiënten minder mobiel zijn en daardoor minder kunnen werken en/of deelnemen aan de maatschappij. Tot op heden is het onduidelijk wat de precieze oorzaak van OA is, alhoewel verschillende risicofactoren zijn geïdentificeerd, zoals leeftijd, (over)gewicht en een aantal genetische factoren. Bovendien zijn er ook nog geen adequate medicijnen om de ontwikkeling van OA tegen te gaan. Samen met bewegingsadviezen, worden slechts algemene pijn- en ontstekingsremmers gebruikt en wordt uiteindelijk bij verregaande OA een gewrichtsvervangende operatie uitgevoerd.

Ontsteking tijdens OA

De rol van ontsteking bij OA is onderbelicht. Waar vroeger werd gedacht dat OA vooral mechanische slijtage van het kraakbeen was, komt er nu steeds meer bewijs dat ontsteking ook een belangrijke rol bij de initiatie en mogelijk beloop van OA speelt. Ontsteking kan zich voordoen in de binnenbekleding van het gewricht, het

synovium, waar zich verschillende ontstekingscellen bevinden. Ondanks dat de mate van deze ontsteking over het algemeen lager is dan bij autoimmuunziektes zoals reumatoïde artritis, wordt bij zeker 50% van de OA patiënten ontsteking in het synovium vastgesteld met behulp van arthroscopie. Verder onderzoek heeft laten zien dat synoviale ontsteking voorspellend is voor kraakbeenschade. Op cellulair en moleculair niveau zijn er ook voldoende aanwijzingen dat ontsteking een rol speelt bij OA. Ontstekingscellen zoals macrofagen worden in grote mate aangetroffen in het synovium van OA patiënten en wanneer macrofagen uit het gewricht worden verwijderd bij muizen, ontwikkelen deze minder ernstige OA. Ontstekingsmediatoren, zoals de cytokines, worden ook in verhoogde concentraties gevonden in het synovium van OA patiënten en verschillende dierstudies laten zien dat deze stoffen een rol spelen bij de ontwikkeling van OA.

DAMPs en S100-eiwitten tijdens ontsteking

Naast cytokines zijn er ook andere ontstekingsstoffen die vrij kunnen komen bij OA. De zogenaamde alarmines of DAMPs (damage associated molecular patterns) komen vrij bij schade of ontsteking en kunnen andere (ontstekings) cellen waarschuwen en activeren. Net als cytokines zorgen ze dan voor verdere ontsteking, die in het geval van OA nadelig kan zijn voor de kwaliteit van het kraakbeen. Belangrijke DAMPs zijn de eiwitten S100A8 en S100A9. Het zijn eiwitten van de familie van S100-eiwitten, zo'n twintigtal eiwitten die verschillende functies binnen en buiten de cel kunnen hebben. S100A8/S100A9 (afgekort tot S100) kan in grote hoeveelheden worden geproduceerd door ontstekingscellen, zoals macrofagen, maar ook granulocyten en monocyten. Studies waarbij S100 gebruikt is om cellen te stimuleren, laten zien dat deze DAMP een groot scala aan ontstekingsmediatoren kan induceren. S100 is ook verhoogd aangetroffen bij een groot aantal (auto)immuunziektes waarbij ontsteking een essentiële rol speelt, zoals dermatomyositis, SLE (systemic lupus erythematosus), arteriosclerose, psoriasis, de ziekte van Crohn en verschillende vormen van artritis, waaronder de "klassieke" reuma (reumatoïde artritis). Associaties van S100 met OA zijn tot op heden zeer schaars. Een enkele studie laat zien dat S100 aanwezig is tijdens een muismodel van OA, maar geeft verder geen verklaringen of effecten hiervan.

Doel van het proefschrift

In dit proefschrift hebben we onderzoek gedaan naar de rol van ontsteking en S100 bij OA. We hebben hiervoor gebruikt gemaakt van weefselmateriaal van OA patiënten, een grootschalig cohort (grote groep deelnemers/patiënten met vergelijkbare kenmerken) van patiënten met symptomatische OA en verschillende muismodellen voor OA. Vervolgens hebben we via het gebruik van ontstekingsremmende cel-therapie en het blokkeren van S100 getracht de synoviale ontsteking tijdens OA in de muis te verlagen en daarmee OA ontwikkeling te stoppen.

Ontsteking en S100 dragen bij aan schade bij muismodellen voor OA

In hoofdstuk 2 hebben we eerst de spiegels van S100 in twee verschillende muismodellen voor OA bestudeerd. Collagenase-geïnduceerde osteoartritis (CIOA) (opgewekt door het inspuiten van collagenase direct in het kniegewricht) is een model dat een duidelijke synoviale ontsteking heeft en het chirurgische DMM (waarbij het ligament tussen de meniscus en het scheenbeen wordt doorgesneden en er instabiliteit ontstaat in het kniegewricht) heeft aanzienlijk minder ontsteking. Beide modellen ontwikkelen duidelijke kraakbeenschade, waarbij het ziekteverloop van de CIOA iets sneller is (maximale schade na 42 dagen) dan bij DMM (56 dagen). In het CIOA model vonden we dat S100 significant verhoogd was tot 21 dagen na de start van het model, terwijl andere ontstekingsmediatoren zoals IL-1β en TNFα na één week al sterk verlaagd waren. In het DMM model was het niveau van S100 beduidend lager in vergelijking met CIOA en slechts licht verhoogd op dag 7. Vervolgens hebben we beide modellen opgewekt in muizen die door een genetisch defect geen S100A9 kunnen aanmaken en daardoor eveneens geen stabiel en functioneel S100A8 kunnen produceren. Deze S100-knock-out (k.o.) muizen hadden een sterk verlaagde synoviale ontsteking en kraakbeenschade in het CIOA model, terwijl dit niet verschilde in het DMM model.

In hoofdstuk 3 hebben we wederom CIOA en DMM geïnduceerd in de S100-k.o. muizen en ons ditmaal geconcentreerd op de formatie van osteofyten. Dit zijn nieuw gevormde kraakbeen/bot structuren op plaatsen in het gewricht waar

ze niet thuishoren. Deze structuren limiteren beweging van het gewricht en veroorzaken pijn tijdens OA. Bij het DMM model, met lage synoviale ontsteking, vonden we geen verschillen tussen normale en S100-k.o. muizen. Echter, halverwege (dag 21) en op het eindpunt (dag 42) van het CIOA model was de omvang van de osteofyten in de S100-k.o. muizen drastisch kleiner.

Deze gecombineerde resultaten geven aan dat ontsteking een belangrijke bijdrage levert aan OA-gerelateerde kraakbeenschade en osteofyt-vorming en dat deze ontsteking deels via S100 gereguleerd wordt.

S100 voorspelt mogelijk ontwikkeling van schade bij beginnende OA patiënten

Daarnaast hebben we in hoofdstuk 2 en 3 onderzoek gedaan naar de relatie tussen S100 en beginnende OA. Hiervoor hebben we het CHECK (cohort heup en cohort knie) cohort gebruikt. Dit Nederlandse cohort gestart door het reumafonds en verspreid over 14 centra beschikt over uitgebreide data van patiënten met symptomatische OA die nog geen verleden van gewrichtsziekten of andere immunologische aandoeningen hebben, tussen de 45 en 65 jaar zijn, en die voor het eerst bij de huisarts komen met knie- of heupklachten. Deze patiënten zijn tot 5 jaar na de eerste klachten gevolgd.

Niveaus van zowel S100A8 als S100A9 in het synovium van CHECK patiënten waren duidelijk verhoogd in vergelijking met die van gezond synovium. Verder vonden we dat hoge spiegels van S100 in het bloed van deze patiënten voorspellend waren voor het ontwikkelen van gewrichtsschade na 2 jaar en het verder ontwikkelen van osteofyten na 2 en 5 jaar. Ondanks dat bij hoge bloedwaardes van S100 niet met zekerheid kan worden vastgesteld dat gewrichtsschade ontstaat, geeft een S100 waarde boven de 200 ng/ml wel een 4x verhoogd risico op (verdere) ontwikkeling van osteofyten. In tegenstelling tot S100 waren enkele standaard bloed-parameters voor ontsteking (C-reactief eiwit (CRP), bezinkingssnelheid (BSE) of COMP (cartilage oligomeric matrix protein)) juist niet voorspellend voor verdere osteofyt ontwikkeling. Deze resultaten wijzen er op dat S100 een unieke vroege biomarker zou kunnen zijn voor de voorspelling van OA ontwikkeling.

S100 heeft catabole en pro-inflammatoire effecten op kraakbeen- en ontstekingscellen

In hoofdstuk 4 en 5 hebben we gekeken naar de effecten van S100 op de verschillende cellen die zich in het gewricht bevinden en die een rol spelen bij de ontwikkeling van OA. Ten eerste hebben we in hoofdstuk 4 de effecten onderzocht van S100 op chondrocyten (kraakbeencellen). Stimulatie van chondrocyten afkomstig van OA patiënten met S100A8 of S100A9, veroorzaakte een sterke verhoging van allerlei ontstekingsmediatoren (voornamelijk cytokines IL-1β, -6 en -8) op zowel RNA als eiwit niveau. Bovendien resulteerde S100 stimulatie in verhoogde spiegels van enzymen die kraakbeen kunnen afbreken, de MMP's (matrix metalloproteinases), en verlaagde niveaus van groeifactoren, eiwitten die juist voor kraakbeenopbouw zorgen. Tenslotte toonden we in hoofdstuk 4 aan dat S100 veel potenter werkt op OA chondrocyten in vergelijking met normale chondrocyten. Samengevat laten we zien dat S100 vooral op chondrocyten van OA patiënten ontstekingsbevorderende (pro-inflammatoire) en kraakbeenafbrekende (catabole) effecten heeft.

Het synovium bevat verschillende typen cellen. Bindweefselcellen (=fibroblasten) zitten voornamelijk in de diepere lagen en hebben een relatief lage activiteit. Macrofagen, belangrijke cellen van het aspecifieke immuunsysteem, bevinden zich voornamelijk in de binnenste laag van het synovium, grenzend aan de holte en in direct contact met de synoviale vloeistof waarin (ontstekings)mediatoren worden uitgescheiden die kraakbeen en bot kunnen beïnvloeden. Macrofagen kunnen worden onderverdeeld in pro- en anti-inflammatoire subtypes genaamd M1- en M2-macrofagen. In hoofdstuk 5 onderzochten we de effecten van S100 stimulatie op intact synovium van OA patiënten en de verschillende celtypen die daarin voorkomen. Eerst stimuleerden we kleine synovium biopten, met S100A8 en S100A9. Net als bij de chondrocyten, werden ook hier pro-inflammatoire cytokines zoals IL-1β, -6 en -8 en catabole MMPs duidelijk verhoogd. S100 had echter geen effecten op fibroblasten uit dit OA synovium. Stimulatie van zowel de pro- als de anti-inflammatoire M1- en M2-macrofagen met S100 zorgde wél voor een toename van pro-inflammatoire cytokines. Uit hoofdstuk 4 en 5 concluderen we dat S100, in grote mate aanwezig tijdens synoviale ontsteking, een pro-inflammatoir en catabool milieu kan stimuleren in het gewricht tijdens OA.

S100-remmer paquinimod vermindert OA-schade in de muis

In de laatste drie hoofdstukken hebben we ons gefocust op het remmen van synoviale ontsteking tijdens OA in de muis, als modelsysteem voor de mens. In hoofdstuk 6 hebben we gebruik gemaakt van paquinimod, een specifieke remmer van S100A9. Deze stof is eerder met succes getest in muismodellen van verschillende immuunziekten en is bovendien in een aantal kleine patiënten-studies veilig gebleken voor de mens. Wanneer we paquinimod enkele dagen voor de inductie van CIOA toedienden aan de muizen, zagen we significante verlagingen van de synoviale ontsteking, osteofyt-grootte en kraakbeenschade na 42 dagen. Paquinimod behandeling van het DMM model, met een lage ontstekingsgraad van het synovium, leverde nagenoeg geen verbetering in de OA ontwikkeling op. Vervolgens lieten we zien dat paquinimod duidelijk pro-inflammatoire en catabole effecten van S100 op synovium van OA patiënten blokkeerde. Deze resultaten, samen met het feit dat paquinimod tot nu toe nauwelijks bijwerkingen heeft laten zien in patiënten trials, levert een mogelijk interessante therapeutische optie voor OA patiënten met aanzienlijke synoviale ontsteking.

Vetstamcellen verlagen ontsteking en S100 en daarmee OA-schade in de muis

In hoofdstukken 7 en 8 tenslotte, hebben we synoviale ontsteking verlaagd door het gebruik van adipose mesenchymale stamcellen (ASCs), stamcellen die afkomstig zijn uit vetweefsel. Van deze cellen is bekend dat ze naast de mogelijkheid tot het ontwikkelen naar chondrocyten, vetcellen en botcellen, ook potente anti-inflammatoire eigenschappen hebben. ASCs kunnen een scala aan anti-inflammatoire mediatoren produceren en zijn al gebruikt in verschillende (auto)immuunziekten. Wanneer we ASCs vroeg na inductie van CIOA lokaal in de knie injecteerden, zagen we significant gereduceerde synoviale ontsteking, osteofyt vorming en kraakbeenschade na 42 dagen. Diepgaandere analyse van de moleculaire effecten van ASCs in het synovium, toonden aan dat een lokale behandeling met deze cellen al na 6 uur pro-inflammatoire cytokines zoals IL-1β en DAMPs S100 verlaagden. Daarna injecteerden we stamcellen in OA muismodellen met verschillende gradaties van synoviale ontsteking en vonden

dat ASCs effectiever waren met toenemende ontsteking. Tenslotte vonden we dat S100 spiegels in het bloed na lokale behandeling van het CIOA kniegewricht met ASCs sterk werd verlaagd, hetgeen er op duidt dat bloedspiegels van S100 een succesvolle behandeling weerspiegelen.

Eindconclusie

In dit proefschrift hebben we aangetoond dat synoviale ontsteking en de resulterende S100 productie essentieel zijn bij de ontwikkeling van kraakbeenschade en osteofyt-vorming tijdens OA. Verder laten veelbelovende data in OA muismodellen zien dat het blokkeren van synoviale ontsteking met behulp van anti-inflammatoire adipose stamcellen of met paquinimod (daarbij S100 blokkerend) zeer interessante toekomstige opties zijn voor de behandeling van OA gekenmerkt door veel ontsteking.

Curriculum vitae

Rik Frederik Pieter Schelbergen werd op 15 december 1981 geboren te Venray en groeide op in Grubbenvorst. Hij rondde in 2000 zijn VWO diploma af aan het St. Thomascollege (later Valuascollege) te Venlo.

Direct hierna ging hij Biologie studeren aan de Katholieke Universiteit Nijmegen (later Radboud Universiteit). Tijdens zijn masterfase liep hij achtereenvolgens stage bij de afdeling Moleculaire Dierfysiologie van de Radboud Universiteit en bij GlaxoSmithKline in Stevenage, Engeland. In september 2006 studeerde hij succesvol af met als afstudeerrichting Medische Biologie. Tijdens zijn laatste jaar en na het afstuderen vervulde hij met veel plezier een aantal jaren onderwijstaken als student-assistent bij practica en werkcolleges in het 1e, 2e en 3e jaar van diezelfde Biologie opleiding.

Na een 3,5 maand durende reis door Centraal-Amerika, begon hij in september 2007 aan de post-doctorale lerarenopleiding biologie aan het Instituut voor Leraar en School (later Radboud Docenten Academie) aan de Radboud Universiteit Nijmegen. Hierbij liep hij driekwart jaar stage op het Lindenholt College (later Mondialcollege) in Nijmegen. Na een uitdagend en intensief jaar, behaalde hij in september 2008 zijn 1e graads bevoegdheid als docent biologie.

Vervolgens begon hij op 1 januari 2009 als promovendus bij de afdeling Reumatologie van het UMC St. Radboud ziekenhuis (later Radboud umc) in Nijmegen. Hier deed hij ruim vijf jaar laboratorisch onderzoek naar de rol van ontsteking en de DAMPs S100A8 en S100A9 bij artrose onder leiding van professor Wim van den Berg en directe begeleiding van Dr. Peter van Lent. De resultaten hiervan staan beschreven in dit proefschrift.

Inmiddels is Rik sinds half augustus 2014 gaan werken als Docent Life Sciences bij de Hogeschool Arnhem en Nijmegen (HAN).

List of publications

Cremers NA, Lundvig DM, van Dalen SC, **Schelbergen RF**, van Lent PL, Szarek WA, Regan RF, Carels CE, Wagener FA.
Curcumin-induced heme oxygenase-1 expression prevents H_2O_2-induced cell death in wild type and heme oxygenase-2 knockout adipose-derived mesenchymal stem cells. *International Journal of Molecular Sciences, 2014 Oct, 15(10), 17974-99*

Schelbergen RF, de Munter W, van den Bosch MH, Lafeber FP, Sloetjes A, Vogl T, Roth J, van den Berg WB, van der Kraan PM, Blom AB, van Lent PL**.**
Alarmins S100A8/S100A9 aggravate osteophyte formation in experimental osteoarthritis and predict osteophyte progression in early human symptomatic osteoarthritis. *Annals of the Rheumatic Diseases, 2014 Sep, Epub ahead of print*

Schelbergen RF, van Dalen S, ter Huurne M, Roth J, Vogl T, Noël D, Jorgensen C, van den Berg WB, van de Loo FA, Blom AB, van Lent PL.
Treatment efficacy of adipose-derived stem cells in experimental osteoarthritis is driven by high synovial activation and reflected by S100A8/A9 serum levels. *Osteoarthritis Cartilage, 2014 Aug, 22(8), 1158-66*

Hofkens W, **Schelbergen RF**, Storm G, van den Berg WB, van Lent PL.
Liposomal targeting of prednisolone phosphate to synovial lining macrophages during experimental arthritis inhibits M1 activation but does not favor M2 differentiation. *PLoS One. 2013, 8(2):e54016*

ter Huurne M, **Schelbergen RF**, Blattes R, Blom A, de Munter W, Grevers LC, Jeanson J, Noël D, Casteilla L, Jorgensen C, van den Berg W, van Lent PL.
Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. *Arthritis & Rheumatism, 2012 Nov, 64(11), 3604-13*

van Lent PL, Blom AB, **Schelbergen RF**, Sloetjes A, Lafeber FP, Lems WF, Cats H, Vogl T, Roth J, van den Berg WB.
Active involvement of alarmins S100A8 and S100A9 in the regulation of synovial

activation and joint destruction during mouse and human osteoarthritis. *Arthritis & Rheumatism, 2012 May, 64(5), 1466-76*

Schelbergen RF, Blom AB, van den Bosch MH, Sloetjes A, Abdollahi-Roodsaz S, Schreurs BW, Mort JS, Vogl T, Roth J, van den Berg WB, van Lent PL.
Alarmins S100A8 and S100A9 elicit a catabolic effect in human osteoarthritic chondrocytes that is dependent on Toll-like receptor 4. *Arthritis & Rheumatism, 2012 May, 64(5), 1477-87*

van Lent PL, Grevers LC, **Schelbergen RF**, Blom A, Geurts J, Sloetjes A, Vogl T, Roth J, van den Berg WB
S100A8 causes a shift toward expression of activatory Fcγ receptors on macrophages via toll-like receptor 4 and regulates Fcγ receptor expression in synovium during chronic experimental arthritis. *Arthritis & Rheumatism, 2010 Nov, 62(11), 3353-64*

Dankwoord

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